Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/001275

International filing date: 14 January 2005 (14.01.2005)

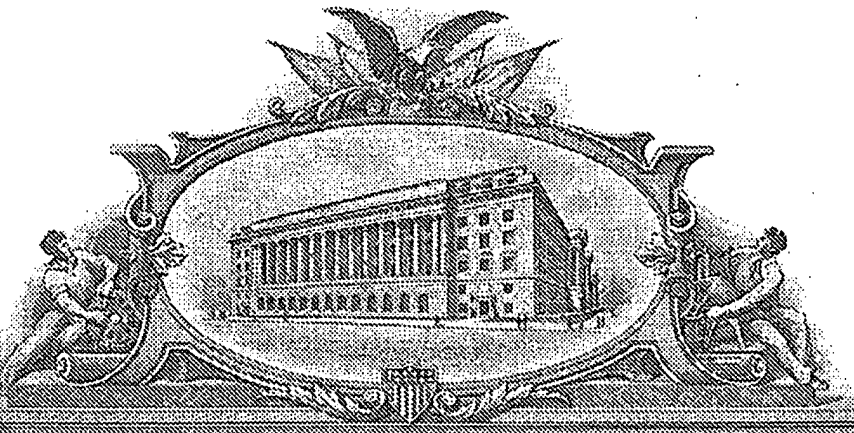
Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/537,053
Filing date: 16 January 2004 (16.01.2004)

Date of receipt at the International Bureau: 03 March 2005 (03.03.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse



1287450

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

February 22, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/537,053*
FILING DATE: *January 16, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/01275*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

I hereby certify that this Provisional Application is being deposited as Express Mail (Express Mailing Label No. EV 306 725 332 US with the U.S. Postal Service in an envelope addressed to: Mail Stop Provisional, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on 16 January 2004 .

Carolyn C. Caires

Carolyn C. Caires

PTO/SB/16 (10-01)
Approved for use through10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

011604 14230 U.S.PTO

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a requ st for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mailing Label No. EV 306 725 332 US

00/72 60/537053 U.S.PTO

APPLICANT(S)/INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Stephen J. David Y.	Klaus Liu	San Francisco, California Palo Alto, California

☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

CONNECTIVE TISSUE GROWTH FACTOR SIGNALING

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number ☐ OR *Type Customer Number here* → *Place Customer Number Bar Code Label here*

☒ Firm or Individual Name	Christopher Turner, Ph.D.				
Company	FibroGen, Inc.	Dept.	Intellectual Property Dept.		
Address	225 Gateway Blvd.				
City	South San Francisco	State	CA	ZIP	94080
Country	US	Telephone	650-866-7200	Fax	650-866-7292

ENCLOSED APPLICATION PARTS ***(check all that apply)***

☒ Specification *Number of Pages* 17
☒ Drawing(s) *Number of Sheets* 8
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☐ Other (specify)

METHOD OF PAYMENT OF FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the fees
☒ The Commissioner is hereby authorized to charge any fee due or credit any overpayment to Deposit Account Number: 50-0811
☐ Payment by credit card. Form PTO-2038 is attached.

FEE AMOUNT ($) 80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME Christopher Turner, Ph.D.

TELEPHONE 650-866-7200

Date 16 Jan. 2004

REGISTRATION NO. 45,167

Docket No.: FP0815 P

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

I hereby certify that this Provisional Application is being deposited as Express Mail (Express Mailing Label No. EV 306 725 332 US) with the U.S. Postal Service in an envelope addressed to: Mail Stop Provisional, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on 16 January 2004.

Carolyn C. Caires

Carolyn C. Caires

PTO/SB/17 (01-03)
Approved for use through 04/30/2003. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

FEE TRANSMITTAL for FY 2003

Effective 01/01/2003. Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
U.S. Serial No.	To be assigned
Filing Date	Concurrently herewith
Applicant(s)	Klaus and Liu
Examiner Name	To be assigned
Art Unit	To be assigned
Docket No.	FP0815 P

METHOD OF PAYMENT *(check all that apply)*

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: 50-0811

Deposit Account Name: FibroGen, Inc.

The Commissioner is authorized to: *(check all that apply)*

☒ Charge any fee(s) indicated below ☒ Credit any overpayments

☒ Charge any additional fee(s)

☐ Charge fee(s) indicated below, **except for the filing fee** to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below	Fee Paid
Total Claims		-20** =		x = 0.00
Independent Claims		- 3** =		x = 0.00
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

***or number previously paid, if greater; For Reissues, see above*

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 0.00

SUBMITTED BY				(Complete *(if applicable)*)	
Name (Print/Type)	Christopher Turner, Ph.D.	*Registration No. (Attorney/Agent)*	45,167	*Telephone*	650-866-7200
Signature				*Date*	16 January 2004

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

This collection of information is required by 37 CFR 1.17 and 1.27. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, Washington, DC 20231.**

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

CONNECTIVE TISSUE GROWTH FACTOR SIGNALING

BACKGROUND OF THE INVENTION

Heparan Sulfate Proteoglycans (HSPG)

Heparan sulfate proteoglycans (HSPGs) are components of the extracellular milieu and are classified as either membrane anchored, e.g., glypicans; transmembrane, e.g., syndicans; or cell associated, e.g., perlecan. Additionally, HSPGs include cell membrane proteins such as betaglycan, CD44/epican, and testican. HSPGs consist of a core protein decorated with covalently linked heparan sulfate (HS) chains. (See, e.g., Bernfield et al. (1999) Annu Rev Biochem 68:729-777.) The HS chains are polysaccharides composed of repeating disaccharide units of uronic acid (iduronate or glucuronate) and glucosamine. (Bernfield et al., supra.) The disaccharide units are selectively acetylated at the N position of glucosamine; sulfated at the N, 3-O, and 6-O positions of glucosamine; and/or sulfated at the 2-O position of iduronic acid residues.

HSPGs mediate signaling activities based on the structure and sulfation of their HS chains, which influence interaction with signaling molecules. (See, e.g., Rapraeger (2002) Methods Cell Biol 69:83-109.) For example, specific sulfation of 2-*O* and 6-*O* positions on HS chains is necessary for fibroblast growth factor (FGF) signal transduction. Specifically, the 2-*O* sulfation is required for binding of basic FGF to heparin, and 6-*O* sulfation is required for bFGF dimerization and receptor activation. (Pye et al. (2000) Glycobiology 10:1183-1192; Schlessinger et al. (2000) Mol Cell 6:743-750.) Additional signaling pathways that require HSPGs include Wnt, interferon (IFN)-γ, transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and hepatocyte growth factor. (Reichsman et al. (1996) J. Cell Biol. 135:819-827; Lortat-Jacob et al. (1995) Biochem J 310:497-505; Lyon et al. (1997) J Biol Chem 272:18000-18006; Soker et al. (1994) Biochem Biophys Res Commun 203:1339-1347; and Zioncheck et al. (1995) J Biol Chem 270:16871-16878.)

Sulfation of HS chains is tissue specific, and changes in sulfation have been correlated with regulatory changes in growth factor signaling. (See, e.g., Brickman et al. (1998) J Biol Chem 273:4350-4359; Ai et al. (2003) J Cell Biol 162:341-351.) Mutations that alter HSPG formation, organization, or sulfation lead to defects in signaling pathways. (See, e.g., Forsberg and Kjellen (2001) J Clin Invest 108:175-180; Takei et al. (2004) Development 131:73-82.) Similarly, mutations in enzymes that alter sulfation patterns on HSPGs at the cell surface can lead to modification in cell signaling. (See, e.g., Ai et al., *supra*.)

Connective Tissue Growth Factor (CTGF)

CTGF is a 36 kD, cysteine-rich, heparin-binding, secreted glycoprotein originally isolated from the culture media of human umbilical vein endothelial cells. (Bradham et al. (1991) J Cell Biol 114:1285-1294; Grotendorst and Bradham, USPN 5,408,040.) CTGF belongs to the CCN (CTGF, Cyr61, Nov) family of proteins, which includes the serum-induced immediate early gene product Cyr61, the putative oncogene Nov, the src-inducible gene CEF-10, the Wnt-inducible secreted protein WISP-3, and the anti-proliferative protein HICP/rCOP. (O'Brian et al. (1990) Mol Cell Biol 10:3569-3577; Joliot et al. (1992) Mol Cell Biol 12:10-21; Ryseck et al. (1990) Cell Growth and Diff 2:225-233; Simmons et al. (1989) Proc. Natl. Acad. Sci. USA 86:1178-1182; Pennica et al. (1998) Proc Natl Acad Sci U S A, 95:14717-14722; and Zhang et al. (1998) Mol Cell Biol 18:6131-6141.) CCN proteins are characterized by conservation of 38 cysteine residues that constitute over 10% of the total amino acid content and give rise to a modular structure with N- and C-terminal domains. The modular structure of CTGF includes conserved motifs for insulin-like growth factor binding protein (IGF-BP) and von Willebrand's factor (VWC) in the N-terminal domain, and thrombospondin (TSP1) and a cystine-knot motif in the C-terminal domain.

CTGF expression is induced by members of the Transforming Growth Factor beta (TGFβ) superfamily, which includes TGFβ-1, -2, and -3, bone morphogenetic protein (BMP)-2, and activin, as well as a variety of other regulatory modulators including dexamethasone, thrombin, vascular endothelial growth factor (VEGF), and angiotensin II. (Franklin (1997) Int J Biochem Cell Biol 29:79-89; Wunderlich (2000) Graefes Arch Clin Exp Ophthalmol 238:910-915; Denton and Abraham (2001) Curr Opin Rheumatol 13:505-511; and Riewald (2001) Blood 97:3109-3116.)

Members of the CCN family are expressed upon primary stimulation of a cell, and are thought to modulate subsequent cell signaling events. Although CTGF has been shown to interact with numerous factors including VEGF, TGFβ, insulin-like growth factor (IGF), integrins, and HSPGs, the physiological importance of such interactions is not fully understood. (Inoki et al. (2002) FASEB J 16: 219-221; Abreu et al. (2002) Nat Cell Biol 4: 599-604; Kim et al. (1997) Proc Natl Acad Sci USA 94:12981-12986; Lau and Lam (1999) Exp Cell Res 248:44-57; Gao and Brigstock (2003) J Biol Chem 10.1074/jbc.M313204200.) CTGF expression in healthy tissue is typically low; however, increased expression at both the mRNA and protein level has been correlated with various disorders. One of the strongest correlations exists between CTGF expression and the degree of tissue fibrosis associated with a disorder. (Abraham et al. (2000) J Biol Chem 275:15220-15225; Dammeier et al. (1998) Int J Biochem Cell Biol 30:909-922; diMola et al. (1999) Ann Surg 230(1):63-71; Igarashi et al. (1996) J Invest

Dermatol 106:729-733; Ito et al., supra; Williams et al. (2000) J Hepatol 32:754-761; Clarkson et al. (1999) Curr Opin Nephrol Hypertens 8 :543-548; Gupta et al. (2000) Kidney Int 58:1389-1399; Riser et al. (2000) J Am Soc Nephrol 11:25-38.) CTGF is also expressed at specific times and locations during development and appears to be important in regulating skeletal development. CTGF has also been associated with angiogenesis, potentially regulating both neovascularization during development and angiogenesis associated with wound healing.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, and 1C show dose-dependent adhesion of cells to CTGF presented by epitope-specific anti-CTGF monoclonal antibodies.

Figures 2A, 2B, 2C, and 2D show adhesion of cells to CTGF is dependent on the orientation of CTGF, as defined by epitope-specific anti-CTGF antibodies, and requires CTGF domain 4.

Figures 3A, 3B, and 3C show adhesion of cells to CTGF is dependent on heparan sulfate moieties associated with the adhering cells.

Figure 4 shows binding of CTGF to cells is effectively competed by heparin derivatives containing specific sulfation patterns, but not by derivatives lacking such sulfation.

Figures 5A, 5B, and 5C show adhesion of cells to CTGF can be competed by heparin derivatives containing specific sulfation patterns, but not by derivatives lacking such sulfation.

Figures 6A and 6B show betaglycan directly interacts with CTGF, and betaglycan, TGF-β, and CTGF form a ternary complex associated with cell signaling.

Figure 7 shows CTGF interacts with basic FGF, and that bFGF and betaglycan compete for binding to CTGF.

DESCRIPTION OF THE INVENTION

Before the present compositions and methods are described, it is to be understood that the invention is not limited to the particular methodologies, protocols, cell lines, assays, and reagents described, as these may vary. It is also to be understood that the terminology used herein is intended to describe particular

embodiments of the present invention, and is in no way intended to limit the scope of the present invention as set forth in the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural references unless context clearly dictates otherwise. Thus, for example, a reference to "a fragment" includes a plurality of such fragments, a reference to an "antibody" is a reference to one or more antibodies and to equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications cited herein are incorporated herein by reference in their entirety for the purpose of describing and disclosing the methodologies, reagents, and tools reported in the publications that might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Invention

The present invention provides novel heparan sulfate constructs and compositions involved in binding of connective tissue growth factor (CTGF) to cells and CTGF-mediated cell adhesion. Such heparan sulfate moieties may be present on the surface of a cell or contained within the extracellular matrix, which surrounds cells *in vivo* and *in vitro*. Such heparan sulfate moieties are often present *in vivo* in the form of heparan sulfate proteoglycans (HSPGs). The heparan sulfate moieties may also be present as soluble molecules, e.g., in a form chemically identical or similar to heparin. Such soluble forms are useful as therapeutic agents for use in modulating the association of CTGF with cells, the extracellular matrix, or other components, e.g., growth factors, etc.

The heparan sulfate moieties encompassed in the present invention are generally defined according to their ability to bind CTGF or fragments thereof. Specific fragments of CTGF include the C-terminal half of CTGF, more specifically the domain encoded by exon 5. (See, e.g., International Publication Nos. WO 96/38172 and WO 00/35939.) Additionally, CTGF fragments for use in defining heparan sulfate moieties of the present invention include those described in International Publication No. WO 99/07407; Gao and Brigstock (2003), *supra*; Ball et al. (2003) J Endocrinol 176:R1-7; Ball et al. (1998) Biol Reprod

59:828-835; and Brigstock et al. (1997) J Biol Chem 272:20275-20282; all of which are incorporated by reference herein in their entirety.

In certain aspects, a fragment of CTGF is characterized by the presence of the cystine-knot (CK) domain. Cystine-knot domains are found in various proteins including glycoprotein hormones and extracellular proteins. The C-terminal cystine knot-like domain (CTCK), found in CTGF and several other CCN family members, and other growth factors, e.g., TGFβ, nerve growth factor (NGF), platelet-derived growth factor (PDGF), noggin, and gonadotropin, consists of 2 highly twisted antiparallel pairs of beta-strands containing three disulphide bonds. The domain is non-globular and little is conserved among these presumed homologs except for their cysteine residues. The CT and CTCK domains are predicted to form homodimers. Such proteins containing cystine-knot domains may be used to further characterize heparan sulfate (HS) and/or heparin-like molecules of the invention. Specific molecules may be selected based on selectivity in binding among the various CK-containing proteins; e.g., a molecule may be selected based on its binding to CTGF and other CCN family members, but not other growth factors such as TGF-β, basic FGF (bFGF), etc.; or a molecule may be selected based on its binding to CTGF, but not other CCN family members; etc.

Binding characteristics of any particular HS or heparin-like molecule for use in the present invention can be modified by altering the length, e.g., the number of dissacharide repeats, in the molecule; the charge, e.g., the number of sulfated residues; and/or the charge distribution, e.g., the degree of N-sulfation, 2-*O*-sulfation, and 6-*O*-sulfation on respective sugar residues. Such modifications can be measured for desired characteristics using binding and adhesion assays, as described in the Examples, using CTGF, fragments thereof, and other potential binding proteins, e.g., CK-containing proteins, as described above.

The HS and heparin-like molecules so defined can be utilized to modulate the bioactivity of CTGF. In particular embodiments, the molecule alters CTGF bioactivity by altering the ability of CTGF to interact with a cell surface or an endogenous extracellular matrix-associated HSPG. As other signaling pathways, e.g., bFGF signaling, are known to involve HSPG binding, the present invention specifically provides methods to inhibit the ability of CTGF to interact with HSPG without affecting the activity of other heparin binding growth factors. Such methods comprise administering an HS or heparin-like molecule of the invention to a subject. In these particular embodiments, the molecule is characterized by its ability to inhibit CTGF-mediated cell adhesion or cell binding without affecting the binding or signaling of other factors, e.g., other CCN family members and/or other growth factors such as VEGF or bFGF, as desired.

The present invention also provides specific HSPGs herein identified as CTGF-binding components. In one particular embodiment, the HSPG is betaglycan. As used herein, "betaglycan", also known as "TGF-β type III receptor", is selected from human betaglycan (GenBank Accession No. AAA67061) or an orthologous protein obtained from any other species. (See, e.g., GenBank Accession No. CAB64374; GenBank Accession No. AAC28564; and GenBank Accession No. AAA40813.) Additionally, betaglycan may comprise any fragment of a full-length betaglycan protein, and especially fragments of betaglycan described, e.g., in Lopez-Casillas et al. (1994) J Cell Biol 124(4):557-568; and Pepin et al. (1995) FEBS Lett 377: 368-372; both of which are incorporated by reference herein in their entirety. Further, betaglycan may comprise naturally-occurring or recombinant soluble betaglycan as described, e.g., in Zhang et al. (2001) Immunol Cell Biol 79:291-297; and Vilchis-Landeros et al. (2001) Biochem J 355:215-222, both of which are incorporated by reference herein in their entirety.

Betaglycan is a 349 amino acid transmembrane glycoprotein with a large extracellular region, which binds TGF-β, and a small cytoplasmic region. Betaglycan is considered an "accessory" receptor, since it appears to regulate the interaction of TGF-β with the signaling receptors, TGF-β type I receptor and TGF-β type II receptor, and thus regulate cell stimulation by TGF-β. (See, e.g., López-Casillas et al. (1993) Cell 73:1435-1444; Sankar et al. (1995) J Biol Chem 270:13567-13572; Lastres et al. (1996) J Cell Biol 133:1109-1121; and Sun and Chen (1997) J Biol Chem 272:25367-25372.) The extracellular domain of betaglycan contains heparan and chondroitin sulphate chains; however, it is thought to be the core protein that binds TGF-β isoforms.

The present invention provides methods to modulate growth factor activity mediated by CTGF. For example, the present examples demonstrate that CTGF and TGF-β form a physical complex with betaglycan. As betaglycan is required for proper cell stimulation by TGF-β, in particular embodiments the present invention provides methods to alter TGF-β signaling by inhibiting CTGF interaction with cell surface HSPGs. In certain embodiments, the HSPG is betaglycan.

Further, the present examples demonstrate a novel interaction between CTGF and bFGF, and interactions between CTGF and betaglycan are modulated in the presence of bFGF. In particular embodiments, the invention provides methods to modulate CTGF signaling in conjunction with or mediated by bFGF by blocking the capacity of CTGF to interact with cell surface HSPGs. In certain embodiments, the HSPG is betaglycan.

As described above, members of the CCN family share the domain on CTGF responsible for HSPG interaction. Although the specificity between individual members of the CCN family and respective HSPG moieties may vary, a certain degree of similarity would be expected. The invention, by providing means to identify and distinguish between HS or heparin-like molecules specific for CTGF, and HS or heparin-like molecules generally active against CCN family binding, provides methods that can be used to modulate various CCN family signaling pathways. Therefore, in some embodiments, the invention provides methods to modulate the ability of CTGF to alter signaling by blocking the capacity of CCN family members to interact with cell surface HSPGs. In particular embodiments, the method modulates signaling by Wnt, a developmental and oncogenic factor modulated by CCN family proteins, e.g., Wisp-3. In certain embodiments, the HSPG is associated with activity of the LDL receptor-related protein (LRP).

Recently, it has been demonstrated that betaglycan also binds and regulates the actions of other members of the TGF-β superfamily. For example, betaglycan forms a complex with the type II activin receptor. This complex then binds inhibin A and prevents formation of functional activin type I/II receptor complexes. (See. e.g., Lewis et al. (2000) Nature 404:411–414.) The interaction between inhibin and betaglycan also prevents bone morphogenetic protein (BMP), e.g., BMP-2, BMP-7, and BMP-9, signaling. (See, e.g., Wiater and Vale (2003) J Biol Chem 278:7934-7941.) As CTGF interacts with betaglycan and forms ternary complexes with betaglycan and TGF-β, CTGF may also regulate other facets of betaglycan function. In any case, modifying interactions between betaglycan and signaling factors, e.g., inhibin, using methods of the invention is specifically contemplated. In specific aspects, the invention provides methods to modulate the ability of CTGF to alter activin signaling by blocking the capacity of CTGF to interact with cell surface HSPGs. In other aspects, the invention provides methods to modulate the ability of CTGF to alter inhibin activity by blocking the capacity of CTGF to interact with cell surface HSPGs. In still other aspects, the invention provides methods to modulate the ability of CTGF to alter BMP signaling by blocking the capacity of CTGF to interact with cell surface HSPGs. In particular embodiments, the HSPG is betaglycan.

In all of the embodiments described above, it is a specific aspect of the invention that the degree of inhibition in CTGF binding can be regulated using specific HS or heparin-like molecules. As CTGF has been implicated in pathways that may not involve heparan sulfate, it is envisioned that specific pathways may not be affected by the present procedures. For example, CTGF has been show to interact with integrins, a family of cell adhesion receptors. The present invention contemplates modulation of certain CTGF bioactivities, such as those associated with TGF-β signaling, by altering the ability of CTGF to

interact with cell surface or extracellular matrix-associated HSPGs, without affecting or being affected by, e.g., integrin signaling.

EXAMPLES

The invention will be further understood by reference to the following examples, which are intended to be purely exemplary of the invention. These examples are provided solely to illustrate the claimed invention. The present invention is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only. Any methods that are functionally equivalent are within the scope of the invention. Various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Example 1. Production of recombinant human CTGF (rhCTGF)

A recombinant human CTGF baculovirus construct was produced as described in Segarini et al. (2001, J Biol Chem 276:40659-40667). Briefly, a CTGF cDNA comprising only the open reading frame was generated by PCR using DB60R32 (Bradham et al. (1991) J Cell Biol 114:1285-94) as template and the primers 5' gctccgcccgcagtgggatccATGaccgccgcc 3' and 5' ggatccggatccTCAtgccatgtctccgta 3', which add BamHI restriction enzyme sites to the ends of the amplified product. The native start and stop codons are indicated in capital letters.

The resulting amplified DNA fragment was digested with BamHI, purified by electrophoresis on an agarose gel, and subcloned directly into the BamHI site of the baculovirus PFASTBAC1 expression plasmid (Invitrogen Corp., Carlsbad CA). The sequence and orientation of the expression cassette was verified by DNA sequencing. The resulting CTGF expression cassette was then transferred to bacmid DNA by site-specific recombination in bacteria. This bacmid was then used to generate a fully recombinant CTGF baculovirus in Spodoptera frugiperda SF9 insect cells according to protocols supplied by the manufacturer (BAC-TO-BAC Expression System manual; Invitrogen). Expansion of recombinant baculovirus titers in Sf9 insect cells was performed using standard procedures known in the art.

Hi5 insect cells were adapted for suspension growth by serial passage of cells in shake flask culture accompanied by enrichment at each passage for separated cells. Suspension Hi5 cells were cultured in 1L SF900II SFM media (Invitrogen) supplemented with 20 μg/ml gentamicin (Mediatech, Inc., Herndon VA) and 1x lipid (Invitrogen) in disposable 2.8L Fernbach culture flasks (Corning Inc., Acton MA) on a shaker platform at 110 rpm at 27°C. Once cells reached a density of 1.0-1.5x10^6 cells/ml with a viability

of >95%, they were infected with recombinant baculovirus at a multiplicity of infection (MOI) of 10. The cultures were then incubated at 27°C for an additional 40 to 44 hours. The conditioned media, which contains rhCTGF, was collected, chilled on ice, and centrifuged at 5000 x *g*. The supernatant was then passed through a 0.45 mm filter.

Four liters of conditioned media was loaded over a 5 ml HI-TRAP heparin column (Amersham Biosciences Corp., Piscataway NJ) pre-equilibrated with 50 mM Tris (pH7.5),150 mM NaCl. The column was washed with 10 column volumes of 350 mM NaCl, 50mM Tris (pH 7.5). CTGF was eluted from the column with an increasing NaCl salt gradient. Eluted fractions were screened by SDS-PAGE, and those containing CTGF were pooled.

Heparin purified CTGF was diluted to a final conductivity of 5.7 mS with non-pyrogenic double-distilled water and the pH was adjusted to 8.0. A Q-SEPHAROSE strong anion exchange column (Amersham Biosciences) containing approximately 23 ml resin connected in tandem with a carboxymethyl (CM) POROS polystyrene column (Applied Biosystems) containing approximately 7 ml resin was utilized for endotoxin removal, and capture and elution of purified rhCTGF. Prior to the sample load, the tandem column was washed with 0.5 M NaOH, followed by 0.1 M NaOH, and finally equilibration buffer. The load sample was passed over the tandem column, the Q-Sepharose column was removed, and CTGF was eluted from the CM POROS column (Applied Biosystems) with an increasing 350 mM to 1200 mM NaCl gradient. The purity of the eluted fractions containing CTGF were evaluated by SDS-PAGE analysis before forming a final sample pool.

Example 2. Anti-CTGF Monoclonal Antibodies

2.1 *Antibody Production*

Fully human monoclonal antibodies to human CTGF were prepared using HUMAB mouse strains HCo7, HCo12 and HCo7+HCo12 (Medarex, Inc., Princeton NJ). Mice were immunized by up to 10 intraperitoneal (IP) or subcutaneous (Sc) injections of 25-50 mg recombinant human CTGF in complete Freund's adjuvant over a 2-4 week period. The immune response was monitored by retroorbital bleeds. Plasma was screened by ELISA (as described below), and mice with sufficient titers of anti-CTGF immunogolobulin were used for fusions. Mice were boosted intravenously with antigen 3 and 2 days before sacrifice and removal of the spleen.

Single cell suspensions of splenic lymphocytes from immunized mice were fused to one-fourth the number of P3X63-Ag8.653 nonsecreting mouse myeloma cells (American Type Culture Collection (ATCC), Manassas VA) with 50% PEG (Sigma, St. Louis MO). Cells were plated at approximately $1x10^5$ cells/well in flat bottom microtiter plate and incubated for about two weeks in high-glucose DMEM (Mediatech, Herndon VA) containing L-glutamine and sodium pyruvate, 10% fetal bovine serum, 10% P388D1 (ATCC) conditioned medium, 3-5% ORIGEN hybridoma cloning factor (Igen International, Gaithersburg MD), 5 mM HEPES, 0.055 mM 2-mercaptoethanol, 50 mg/ml gentamycin, and 1x HAT (Sigma). After 1-2 weeks, cells were cultured in medium in which the HAT was replaced with HT. Individual wells were then screened by ELISA (described below). Antibody secreting hybridomas were replated, screened again, and, if still positive for anti-CTGF antibodies, were subcloned at least twice by limiting dilution. The stable subclones were then cultured in vitro to generate small amounts of antibody in tissue culture medium for characterization. One clone from each hybridoma that retained the reactivity of the parent cells was used to generate 5-10 vial cell banks stored in liquid nitrogen.

ELISA assays were performed as described by Fishwild et al. (1996, Nature Biotech 14:845-851). Briefly, microtiter plates were coated with 1-2 μg/ml purified recombinant CTGF in PBS at 50 μl/well, incubated at 4°C overnight, then blocked with 200 μl/well 5% chicken serum in PBS/Tween (0.05%). Dilutions of plasma from CTGF-immunized mice or hybridoma culture supernatants were added to each well and incubated for 1-2 hours at ambient temperature. The plates were washed with PBS/Tween and then incubated with a goat-anti-human IgG F_c polyclonal antibody conjugated with horseradish peroxidase (HRP) for 1 hour at room temperature. After washing, the plates were developed with 0.22 mg/ml ABTS substrate (Sigma) and analyzed by spectrophotometer at 415-495 nm.

2.2 ***Antibody characterization***

Epitope mapping of antibodies by competitive binding experiments is well known by those skilled in the field of immunology. (See, e.g., Van Der Geld et al. (1999) Clinical and Experimental Immunology 118:487-96.) Each antibody population isolated from cells propagated from a unique cloned hybridoma cell was mapped and assigned to a specific binding domain on human CTGF using standard binding and blocking experiments. (See, e.g., Antibodies: A Laboratory Manual (1988) Harlow and Lane (eds), Cold Spring Harbor Laboratory Press; Tietz Textbook of Clinical Chemistry, 2nd ed., (1994) Chapter 10 (Immunochemical Techniques), Saunders; and Clinical Chemistry: Theory, Analysis, Correlation (1984) Chapter 10 (Immunochemical Techniques) and Chapter 11 (Competitive Binding Assays), C.V. Mosby, St. Louis.) For example, epitope mapping was performed by ELISA analysis using specific recombinantly expressed fragments of CTGF. Antibodies that recognized epitopes, e.g., on the N-

terminal domain of CTGF were identified by ELISA analysis against immobilized fragments obtained from recombinant expression of exon 2 and/or exon 3 of the CTGF gene. Antibodies that specifically recognize N-terminal domains or N-terminal fragments of CTGF (e.g., anti-N1, an antibody having specificity for an N-terminal fragment epitope containing CTGF domain 1; anti-N2, an antibody having specificity for an N-terminal fragment epitope containing CTGF domain 2; etc.) or C-terminal domains or C-terminal fragments of CTGF (e.g., anti-C1, an antibody having specificity for a C-terminal fragment epitope containing CTGF domain 3; anti-C2, an antibody having specificity for a C-terminal fragment epitope containing CTGF domain 4; etc.) were selected and utilized in the following examples.

Example 3. Assays

3.1 *Cell Adhesion Assay*

Methods for measuring cell adhesion mediated by CTGF are generally known to those skilled in the art. (See, e.g., Babic et al. (1999) Mol Cell Biol 19:2958-296; Ball et al. (2003) J Endocrinol 176:R1-7.) Such methods typically involve application of CTGF directly to plastic tissue culture plates. Unsaturated protein binding capacity is blocked, e.g., with bovine serum albumin, and wells are seeded with cells. Additional factors, e.g., chelators such as EDTA, peptides, organic compounds, antibodies, etc., may be incubated with the cells prior to plating or added concurrently with the cells. Plates are incubated for a suitable length of time, e.g., 30 to 60 min, at a suitable temperature, e.g., 25 to 37°C, to allow cells to adhere; wells are then washed, and adherent cells are measured. Cell measurements may be made by any method known in the art; e.g., cells may be fixed with formalin, stained, e.g., with methylene blue, and quantified by dye extraction and measurement of absorbance, e.g., at 620 nm.

In a preferred method, tissue culture plates are coated with CTGF indirectly using epitope-specific capture antibodies. In the present examples, cells of a tissue culture plate were coated with a human monoclonal antibody specific for human CTGF, and then were blocked with bovine serum albumin to prevent non-specific binding. Cells were added at a seed density of approximately 8×10^4 cells/well. Additionally, either rhCTGF or fragments thereof, or a vehicle control was added to each well, and the plates were incubated for 45 minutes at 37°C. Wells were then washed, and the number of cells retained in each well was measured using a CYQUANT cell proliferation assay kit (Molecular Probes, Inc., Eugene OR).

In experiments using human dermal foreskin fibroblast cells and a human monoclonal antibody specific for human CTGF domain 3 (anti-C1), dose-sensitive cell adhesion was seen when any of the parameters,

i.e., amount of CTGF, anti-CTGF antibody, or cell number, was altered while the remaining parameters were held constant. For example, a dose-sensitive increase in the number of cells retained in each well was seen when either antibody concentration was held constant (10 μg/ml) and CTGF concentration was increased (Figure 1A), or when CTGF concentration was held constant (2 μg/ml) and anti-CTGF antibody concentration was increased (Figure 1B). Similarly, a dose-sensitive increase in the number of cells retained in each well was seen when cells were titrated in wells coated with a constant amount of antibody (10 μg/ml) and CTGF (2 μg/ml) (Figure 1C).

3.2 *CTGF Binding Assay*

Methods for measuring binding of CTGF to cells are generally known to those skilled in the art. (See, e.g., Nishida (1998) Biochem Biophys Res Commun 247:905-909; Segarini et al. (2001) J Biol Chem 276:40659-40667.) Such methods typically involve labeling CTGF with a detectable moiety, e.g., a radioactive or fluorescent tag, applying the labeled CTGF to cells, washing the cells to remove unbound CTGF, and then measuring the amount of label that remains associated with the cells. Cells may be attached to a surface, e.g., a tissue culture plate, or in suspension. Labeling cells in suspension allows analysis by flow cytometry, e.g. using fluorescently labeled CTGF and a fluorescent-activated cell sorter (FACS).

In a preferred method, cells were suspended in media containing CTGF under conditions suitable for binding of CTGF to cellular targets. Cells may optionally be treated prior to or concurrently with CTGF exposure; for example, cells may be treated with enzymes that alter cell surface moieties, molecules that compete competitively or non-competitively with CTGF for binding to cells, etc. Following incubation to allow CTGF to bind to cells, cells were washed and then incubated with fluorescently-labeled anti-CTGF antibody. The level of CTGF binding was then measured using a FACS apparatus.

3.3 *Co-immunoprecipitation Assay*

Co-immunoprecipitation is a purification procedure used to determine if two different molecules, e.g., proteins, directly interact. Basically, an antibody specific to a protein of interest is added to a cell lysis under conditions suitable for antibody binding to the protein. The antibody-protein complex is then collected, e.g., using protein-G sepharose, which binds most antibodies. Any molecules that are bound to the precipitated protein will also be collected. Identification of proteins can be determined by, e.g., western blot or by direct sequencing of the purified protein(s). Several commercial kits, e.g., the PROFOUND co-immunoprecipitation kit from Pierce Biotechnology, Inc. (Rockford IL) are also available.

In the present examples, co-immunoprecipitations were performed as follows. The surface of intact cells was iodinated with ^{125}I prior to lysing cells and fractionating on a CTGF affinity column. Alternatively, CTGF and labeled cells were incubated for a period sufficient for CTGF binding to cells, and then cells were lysed and immunoprecipitations were performed using anti-CTGF specific antibodies. Antibody complexes were collected from the lysate using protein-G sepharose, and pelleted by centrifugation. Proteins eluted from affinity columns or collected by immunoprecipitation were analyzed by fractionation on SDS-PAGE and visualized by autoradiography. In similar experiments, unlabeled cells or specific proteins were mixed with CTGF alone or in the presence of additional factors, and immunoprecipitations were performed. Following fractionation, proteins were transferred to membranes and probed by western analysis.

Example 4. Regions of CTGF Involved in Cell Binding and Adhesion

4.1 ***Various cell types utilize a similar mechanism in CTGF-mediated adhesion***

The cell adhesion assay described in Example 3.1 was used to identify regions of CTGF involved in cell adhesion. Wells of a tissue culture plate were coated with monoclonal antibodies specific for either amino-terminal domains (anti-N1 or anti-N2 antibodies) or carboxy-terminal domains (anti-C1, -C2) of CTGF (see Figure 2A). HFF were seeded into wells and adhesion was measured as described in Example 3.1.

As shown in Figures 2B, antibodies specific for epitopes associated with the C-terminal domain of CTGF presented CTGF to cells in a manner that facilitated cell adhesion. However, antibodies specific for epitopes on the N-terminal domain of CTGF did not orient CTGF in a manner that allowed cell adhesion.

4.2 ***The C-terminal half of CTGF mediates cell adhesion***

To further define the region of CTGF responsible for cell adhesion, the procedure used in Example 4.1 was further modified as follows. Wells of a tissue culture plate were coated with monoclonal antibodies specific for either amino-terminal (anti-N1) or carboxy-terminal (anti-C1) domains of CTGF (see Figure 2A). Wells were then seeded with HFF in the presence of no CTGF, full-length CTGF, the N-terminal half of CTGF (NH2 fragment), or the C-terminal half of CTGF (COOH fragment). Wells were incubated and adhesion was measured as described in Example 3.1.

Consistent with the results shown in Example 4.1, presentation of full-length CTGF using anti-C1 antibodies facilitated cell adhesion, whereas presentation using anti-N1 antibodies did not (Figure 2C). Further, the C-terminal half of CTGF, when captured using anti-C1 antibodies, was sufficient to provide cell adhesion equivalent to adhesion provided by full-length CTGF. Additionally, the binding was dose responsive, increasing with increasing amounts of CTGF or CTGF fragment. The N-terminal half of CTGF, however, did not provide a suitable substrate for cell adhesion (Figure 2C). The data show that the C- terminal half of CTGF mediates CTGF-dependent adhesion.

4.3 CTGF-dependent adhesion requires domain 4

The cell adhesion assay described in Example 3.1 was used to further define the portion of the C-terminal half of CTGF involved in cell adhesion. Wells of a tissue culture plate were coated with monoclonal antibodies specific for the "hinge" domain of CTGF (anti-H1 antibodies) (see Figure 2A). Wells were then seeded with HFF (8×10^4 cells/well) in the presence of no CTGF, full-length CTGF, or a CTGF construct lacking domain 4 (CTGFΔ4). Wells were incubated and adhesion was measured as described in Example 3.1.

Although HFF were able to adhere to full-length CTGF, they were not able to bind to CTGF lacking domain 4 (CTGFΔ4) (Figure 2D). This result suggests that domain 4, which contains the cystine knot (CK) motif, is necessary for CTGF-mediated cell adhesion.

Example 5. HSPGs are Required for CTGF Binding and CTGF-mediated Adhesion

5.1 Heparan sulfate is involved in CTGF binding and CTGF-dependent cell adhesion

CTGF has been described as a heparin-binding growth factor. As cells may carry a variety of proteoglycan moieties on their surface, e.g., heparan sulfate, chondroitin sulfate, etc. (see Figure 3A), the following experiment was conducted to determine the specificity of CTGF for such moieties. The cell adhesion and cell binding assays were conducted as described in Examples 3.1 and 3.2, respectively, except prior to seeding cells were treated for 1 hour at 37°C with either vehicle, 4 units/ml heparinase I, or 2 units/ml chontroitinase ABC.

As shown in Figure 3B, CTGF-dependent cell adhesion was inhibited by pretreatment of cells with heparinase, but not chondroitinase.

To further examine the requirement for heparan sulfate proteoglycans in CTGF-mediated cell adhesion, adhesion was measured in the presence of increasing amounts of heparin. Heparin and heparan sulphate both consist of repeating disaccharides of uronic acid and glucosamine, but the proportion of N-sulfation of heparan sulfate is typically below 50%, while sulfation of heparin is usually 70% or higher. The cell adhesion assay was conducted as described in Examples 3.1, except increasing concentrations of low molecular weight heparin (LMWH) was additionally added to each adhesion reaction.

As shown in Figure 3C, CTGF-dependent adhesion was inhibited by soluble heparin in a concentration-dependent manner. This result supports the conclusion that CTGF-mediated cell adhesion requires heparan sulfate moieties, i.e., HSPGs.

5.2 ***Differential inhibition of CTGF-mediated cell adhesion and cell binding by modified heparin sulfate oligomers***

The sulfate groups of heparin include 2-O-sulfation of iduronate residues, 6-O-sulfation of iduronate residues, and amino group sulfation (N-sulfation) of glucosamine residues. Sulfates can be selectively removed using chemical methods known to those skilled in the art. Such methods, as described below, can be applied either solely or jointly to obtain a polysaccharide derivative with a desired sulfation pattern. Oligosaccharide libraries can be obtained and screened using methods known to those skilled in the art. (See, e.g., Jemth et al. (2003) J Biol Chem 278: 24371-24376; and Ashikari-Hada et al. (2004) J Biol Chem 10.1074/jbc.M313523200.)

Both O- and N-sulfate groups can be removed, e.g., by heating a pyridinium salt of heparin at 80°C for four hours in dimethylsulfoxide. (See, e.g., Nagasawa et al. (1977) Carbohydr Res 58:47-55.) Since the elimination rate of the N-sulfate group is much greater than that of the O-sulfate group, carrying out the reaction under mild conditions, e.g., reaction at or below 20°C, produces selective de-N-sulfation. (See, e.g., Inoue and Nagasawa (1976) Carbohydr Res 46:87-95.) Sulfate groups can be removed from ether (O-sulfation) linkages under strongly alkaline conditions. The resulting epoxide rings can then be cleaved to yield primarily iduronate residues. Removal of 6-O-sulfation can be carried out, e.g., as described in Takano et al. (1998, Carbohydr Lett 3:71-77).

To determine the specificity of sulfation and charge distribution for CTGF-mediated cell adhesion and cell binding, experiments as described in Examples 3.1 and 3.2, respectively, were performed with the following modification. Combination of HFF cells with CTGF was accompanied by addition of

increasing concentrations of soluble LMWH or heparin sulfate oligomers that were modified to contain differing amounts of sulfation and acetylation covalently bound to either oxygen (O) or nitrogen (N).

As shown in Figure 4, binding of CTGF to HFF requires specific sulfation of heparan sulfate or heparin-like molecules. Specifically, heparin and oversulfated derivatives thereof substantially inhibit CTGF binding to cells. However, de-O-sulfated heparin derivatives were less effective at inhibiting binding, and de-N-sulfation showed no inhibitory capacity. Thus, cell binding by CTGF requires N-sulfation, and is further augmented by both 2-O- and 6-O-sulfation. The dashed line in Figure 4 indicates the level of CTGF binding without any addition of heparin or derivatives. Figures 5A, 5B, and 5C, which show the effect of soluble LMWH or modified heparin sulfate oligomers on CTGF-mediated cell adhesion, confirm the effect of desulfation seen in the CTGF binding assay above.

The data show that there are specific modifications on heparin sulfate that are critical for CTGF binding and cell adhesion, whereas other modifications do not affect CTGF binding or responsiveness. Specifically, the data point to the importance of N-sulfation and O-sulfation of heparin sulfate proteoglycans as being critical for CTGF binding and signaling. These modifications are unique to CTGF and different from modifications known to mediate signaling of other heparin binding growth factors, such as, e.g., bFGF or PDGF. Thus, specific therapeutics can be derived based on heparan sulfate or heparin-like molecules which specifically inhibit CTGF function but do not inhibit the bioactivity of other heparin binding growth factors.

Example 6. Betaglycan is a CTGF-binding HSPG

6.1 *CTGF binds directly to betaglycan*

Identification of cell receptors for CTGF was carried out using co-immunoprecipitation procedures as described in Example 3.3. Initial experiments using radiolabeled cells identified betaglycan as a primary CTGF-binding protein on the cell surface (data not shown). Subsequent experiments using soluble betaglycan (sBetaglycan) demonstrated dose-sensitive interaction between betaglycan and CTGF (Figure 6A). Together, this data shows that betaglycan is a cell surface HSPG that functions as a specific receptor for CTGF.

6.2 *CTGF binds TGFβ and betaglycan in a ternary complex in an HSPG-dependent fashion*

Betaglycan is also known as TGF-β type III receptor and has been shown to facilitate cell stimulation by TGF-β. CTGF has also been associated with TGF-β signaling as an immediate early response factor

produced by cells upon TGF-β signaling. To determine the functional nature of possible interactions between betaglycan, CTGF, and TGF-β, immunoprecipitations were performed as follows. Soluble betaglycan, [^{125}I]-labeled TGF-β, and CTGF were mixed under conditions suitable for interaction, and then complexes were isolated using anti-CTGF antibodies bound to a solid bead matrix. The data show that CTGF, betaglycan and TGF-β form a ternary complex that is dependent on the heparin binding potential of CTGF (Figure 6B). The present invention contemplates that inhibition of ternary complex formation may inhibit betaglycan-dependent CTGF signaling, and may thereby modify TGF-β signaling.

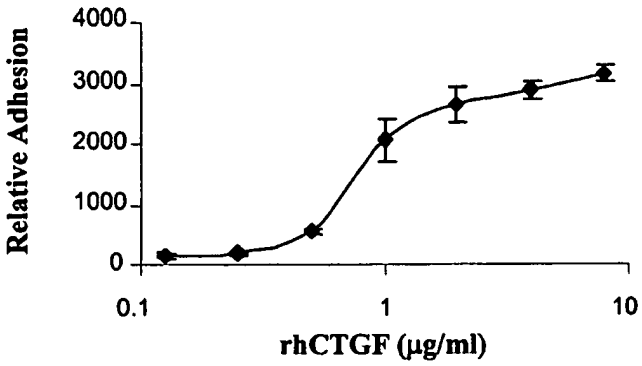
6.3 ***CTGF binds FGF and betaglycan in a ternary complex in an HSPG dependent fashion***

Fibroblast growth factors bind to HSPGs, and signaling by basic and acidic FGF requires this interaction. To determine if the HSPG-dependent interaction between CTGF and betaglycan involves or is modified by FGF, immuno-precipitations were performed as follows. Soluble betaglycan, bFGF, and CTGF were mixed under conditions suitable for interaction, and then complexes were isolated using anti-CTGF antibodies bound to a solid bead matrix. As shown in Figure 7, binding between CTGF and betaglycan is adversely influenced by bFGF in a dose-sensitive manner. Surprisingly, the interaction was not due solely to competition between CTGF and bFGF to heparan sulfate moieties on betaglycan. There was also a clear interaction between CTGF and bFGF, as immunoprecipitation of CTGF in the presence of bFGF, without betaglycan, demonstrated clear interaction between the two growth factors. The result shows that a novel interaction between CTGF and bFGF has been identified, and that selective inhibition of ternary complex formation may inhibit CTGF signaling alone, coordinated signaling between CTGF and TGF-β, and/or coordinated or independent signaling by bFGF.

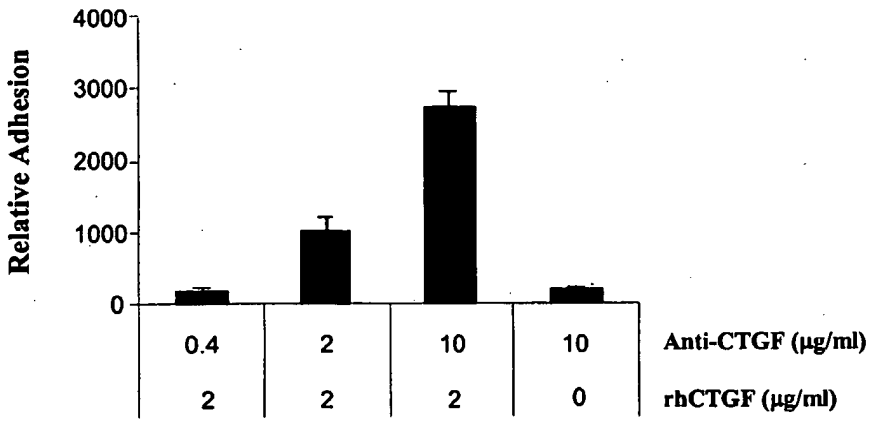
Various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein are hereby incorporated by reference in their entirety.

A.


Relative Adhesion
4000
3000
2000
1000
0
0.1
1
10
rhCTGF (μg/ml)

B.


Relative Adhesion
4000
3000
2000
1000
0
0.4
2
10
10
Anti-CTGF (μg/ml)
2
2
2
0
rhCTGF (μg/ml)

C.

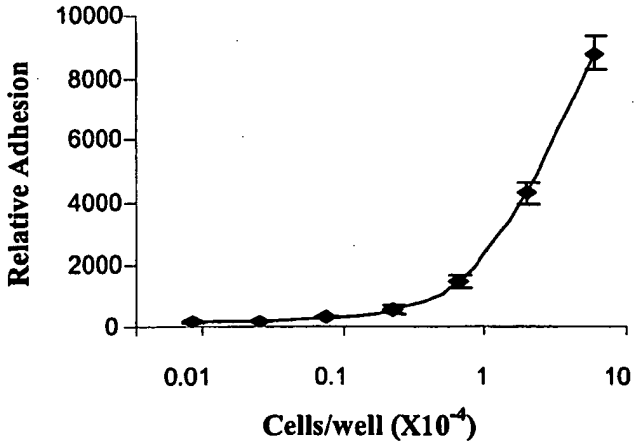

Relative Adhesion
10000
8000
6000
4000
2000
0
0.01
0.1
1
10
Cells/well (X10-4)

Figure 1

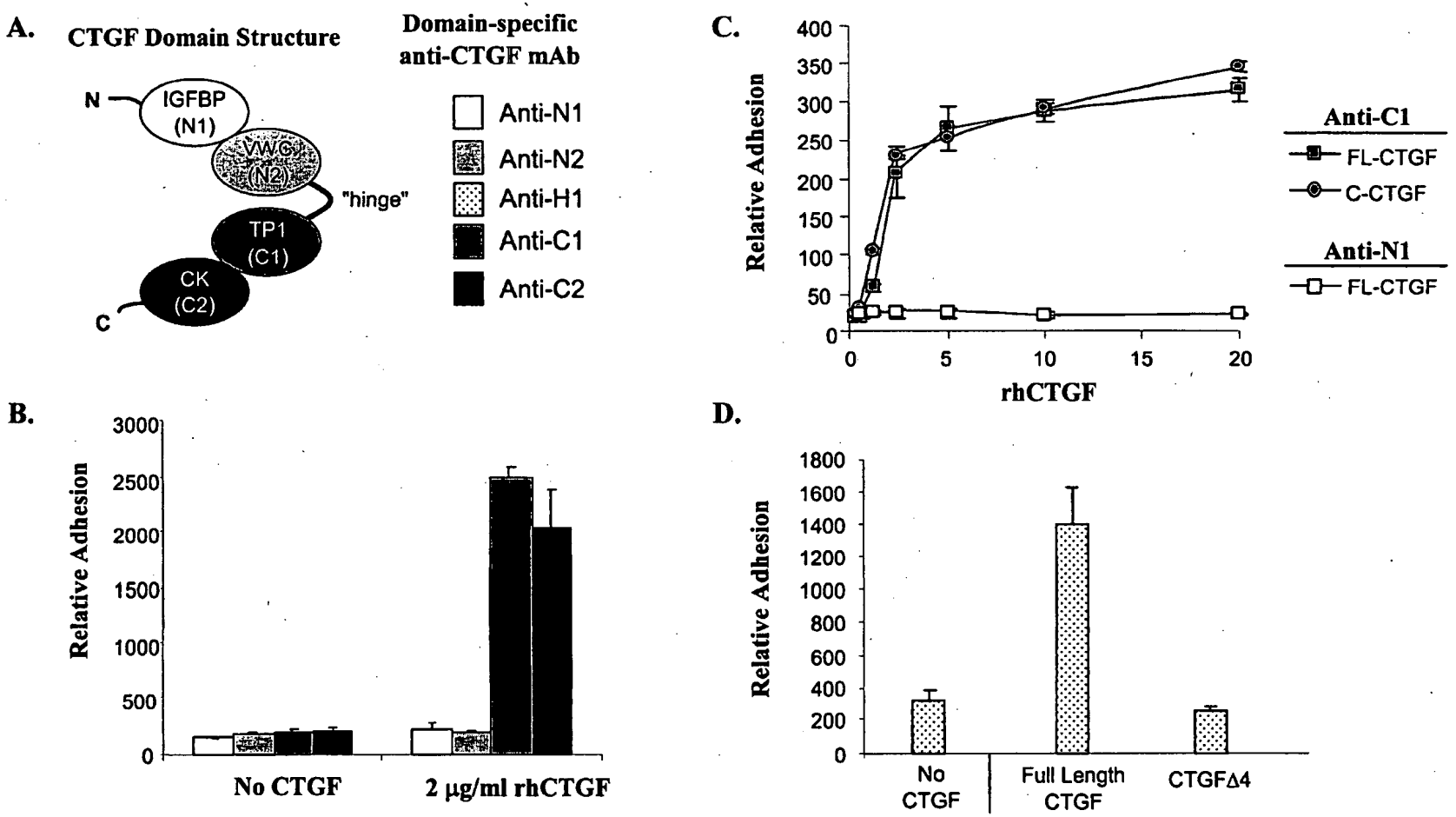
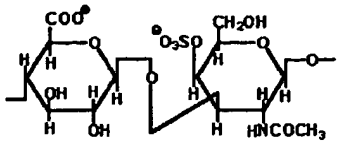
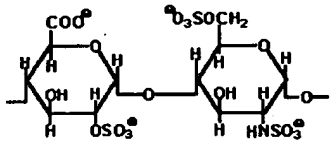

A.
CTGF Domain Structure
Domain-specific anti-CTGF mAb
N
IGFBP (N1)
VWC (N2)
"hinge"
TP1 (C1)
CK (C2)
C
Anti-N1
Anti-N2
Anti-H1
Anti-C1
Anti-C2
B.
Relative Adhesion
3000
2500
2000
1500
1000
500
0
No CTGF
2 µg/ml rhCTGF
C.
Relative Adhesion
400
350
300
250
200
150
100
50
0
0
5
10
15
20
rhCTGF
Anti-C1
FL-CTGF
C-CTGF
Anti-N1
FL-CTGF
D.
Relative Adhesion
1800
1600
1400
1200
1000
800
600
400
200
0
No CTGF
Full Length CTGF
CTGFΔ4

Figure 2

A.

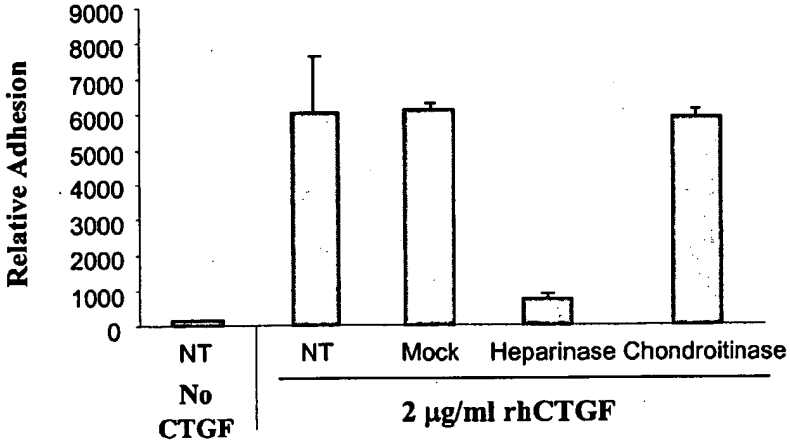


Chondroitin sulfate:
Glucuronate-glucosamine
β (1,3) linkage



Heparan sulfates:
Glucuronate-glucosamine or
Iduronate-glucosamine
α (1,4) linkage

B.



C.

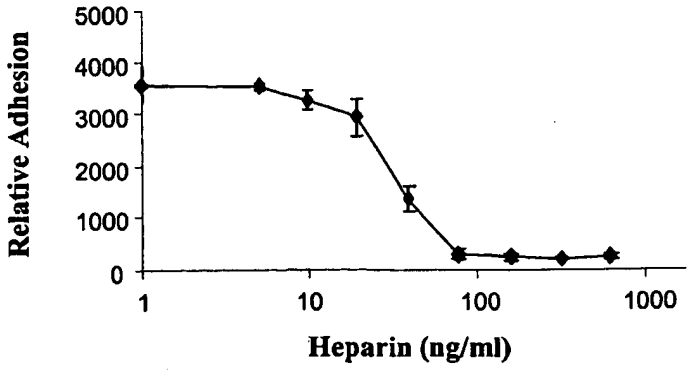


Figure 3

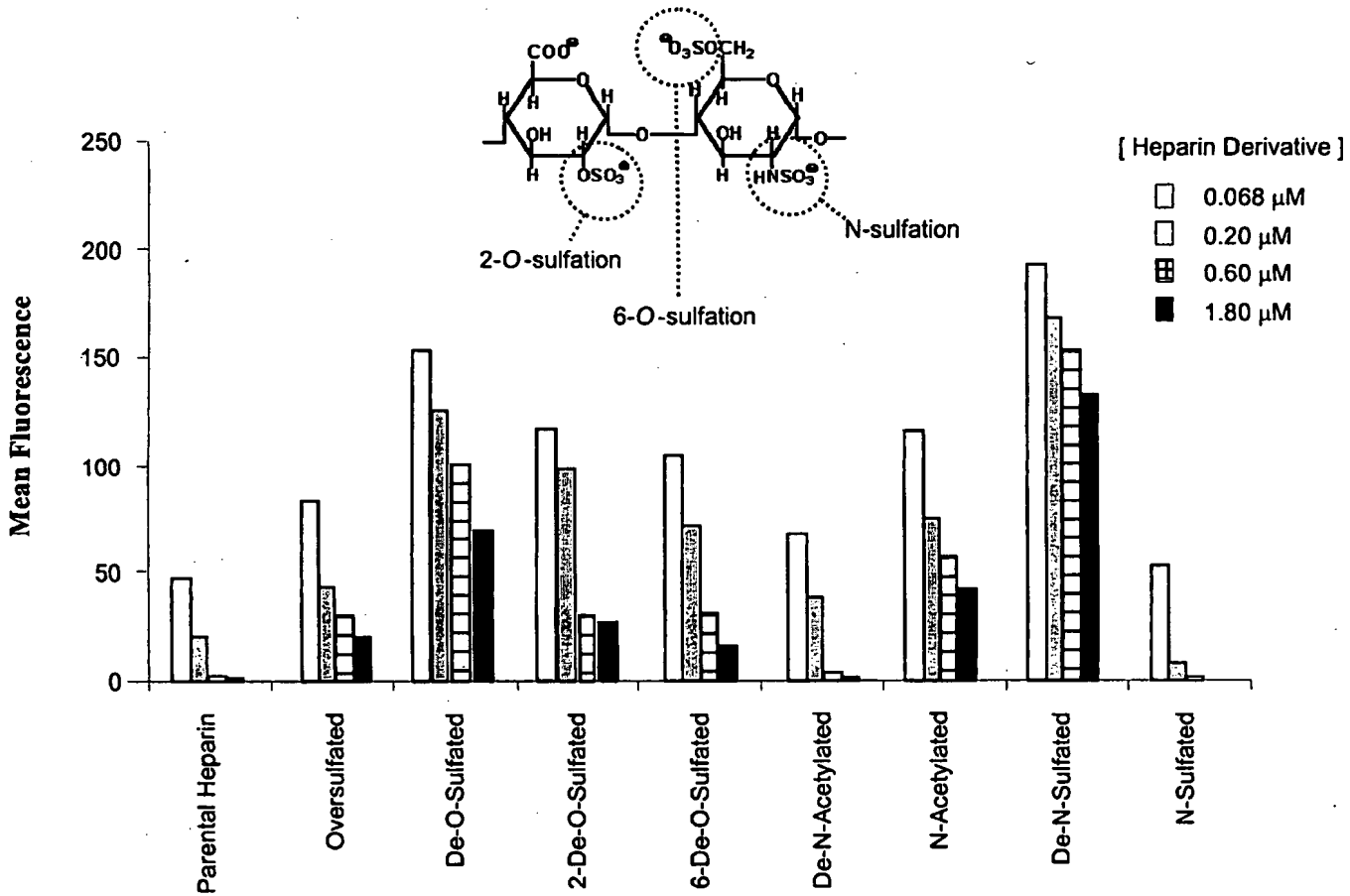

Mean Fluorescence
250
200
150
100
50
0
[Heparin Derivative]
0.068 μM
0.20 μM
0.60 μM
1.80 μM
2-O-sulfation
6-O-sulfation
N-sulfation
Parental Heparin
Oversulfated
De-O-Sulfated
2-De-O-Sulfated
6-De-O-Sulfated
De-N-Acetylated
N-Acetylated
De-N-Sulfated
N-Sulfated

Figure 4

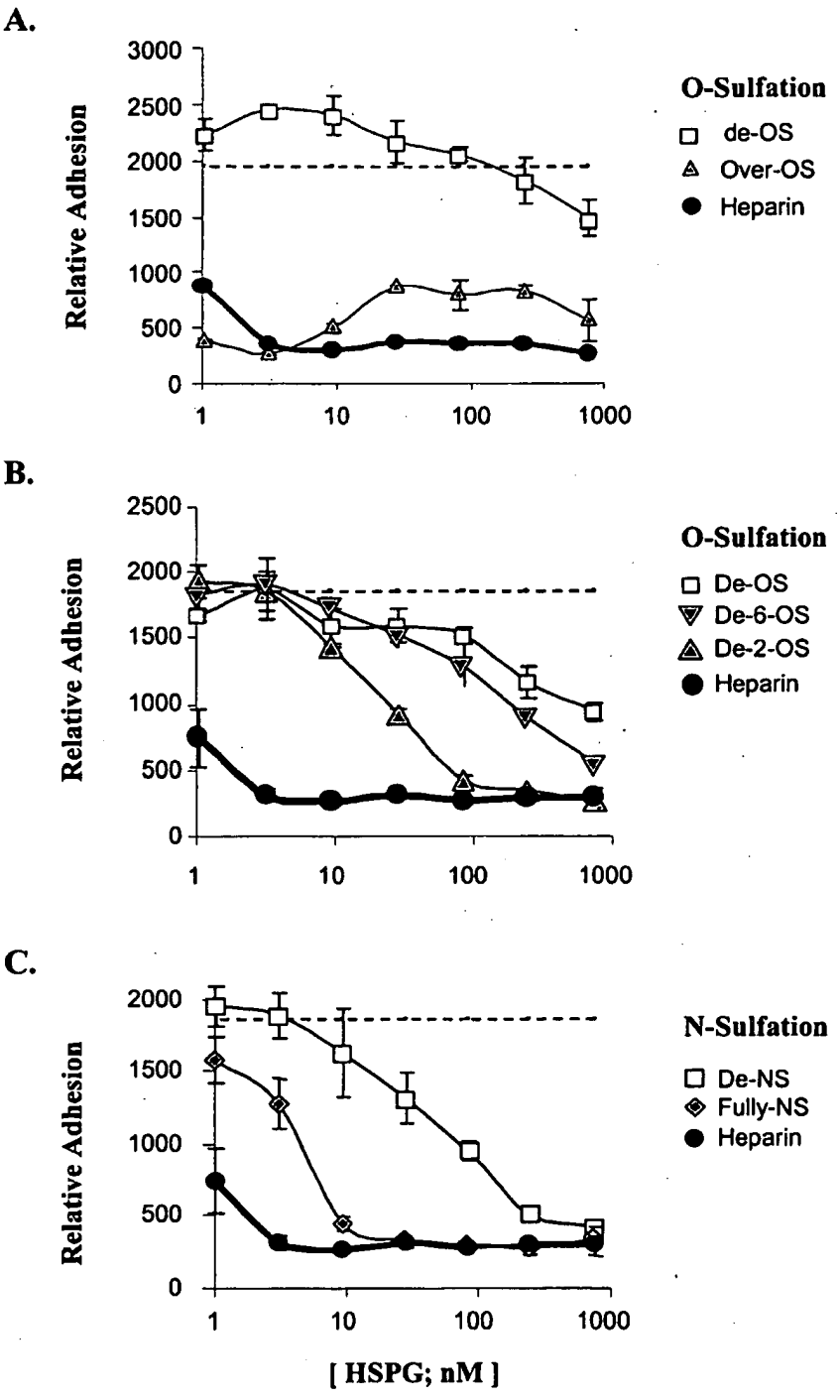
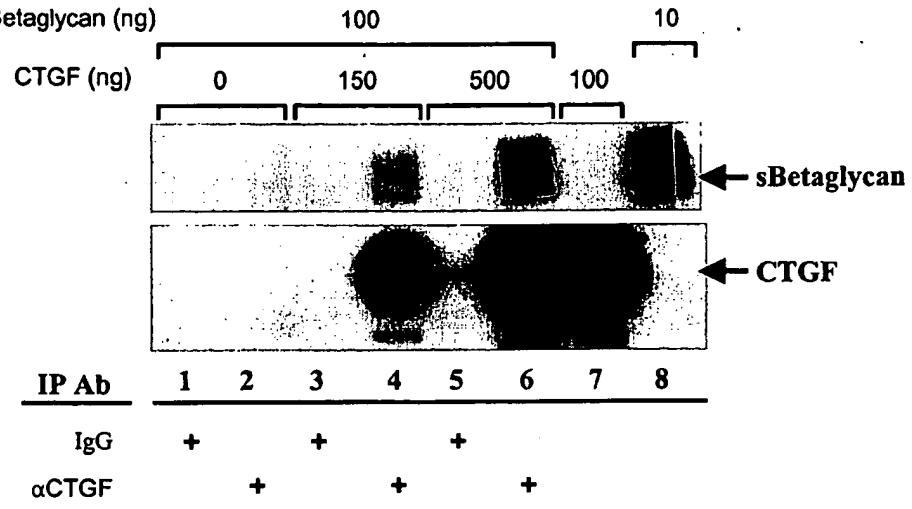

A.
Relative Adhesion
3000
2500
2000
1500
1000
500
0
1
10
100
1000
O-Sulfation
de-OS
Over-OS
Heparin
B.
Relative Adhesion
2500
2000
1500
1000
500
0
1
10
100
1000
O-Sulfation
De-OS
De-6-OS
De-2-OS
Heparin
C.
Relative Adhesion
2000
1500
1000
500
0
1
10
100
1000
[HSPG; nM]
N-Sulfation
De-NS
Fully-NS
Heparin

Figure 5

A.


sBetaglycan (ng)
100
10
CTGF (ng)
0
150
500
100
sBetaglycan
CTGF
IP Ab
1 2 3 4 5 6 7 8
IgG
αCTGF

B.

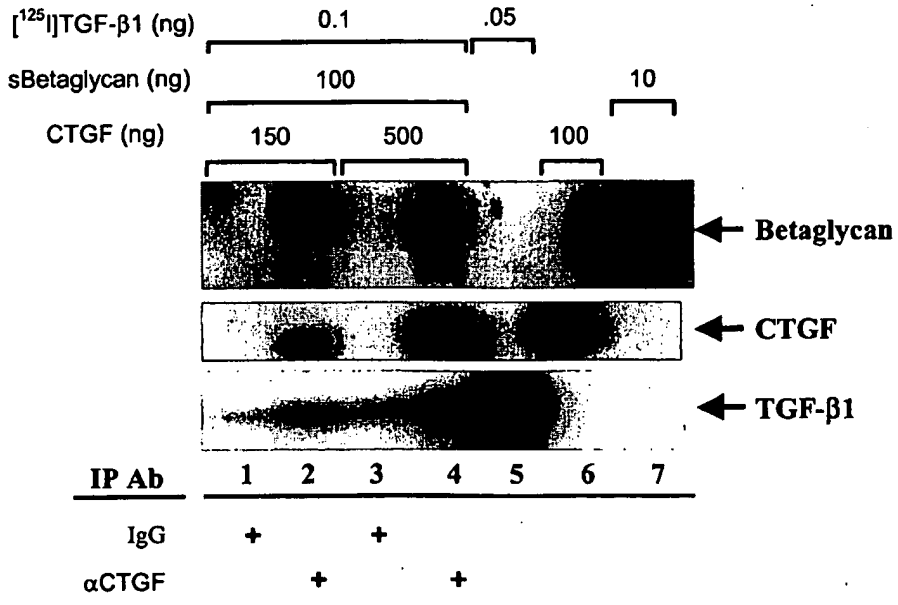

[125I]TGF-β1 (ng)
0.1
.05
sBetaglycan (ng)
100
10
CTGF (ng)
150
500
100
Betaglycan
CTGF
TGF-β1
IP Ab
1 2 3 4 5 6 7
IgG
αCTGF

Figure 6

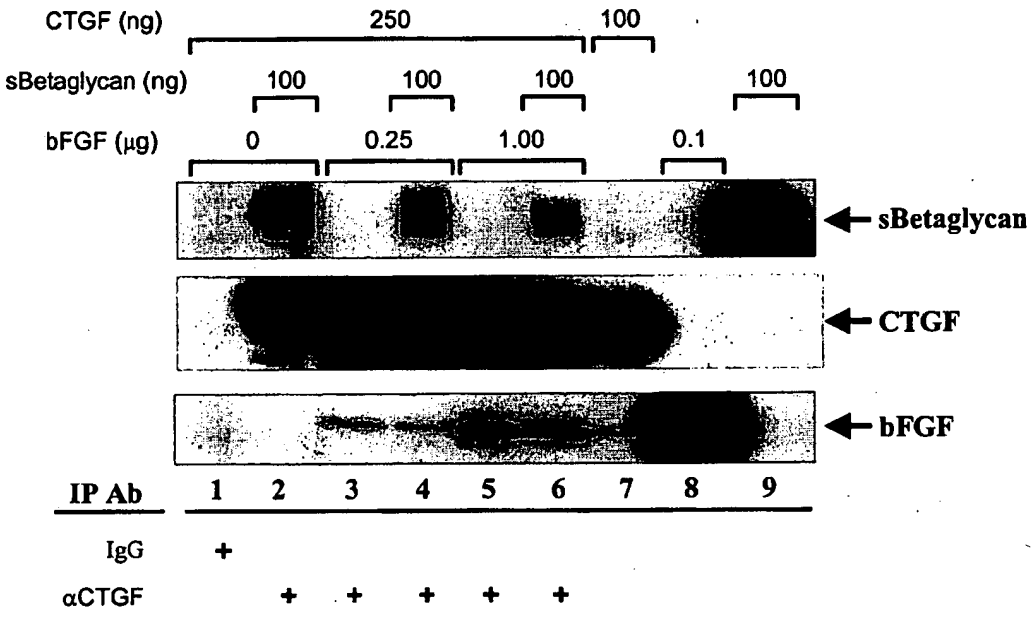

CTGF (ng)
250
100
sBetaglycan (ng)
100
100
100
100
bFGF (μg)
0
0.25
1.00
0.1
sBetaglycan
CTGF
bFGF
IP Ab
1 2 3 4 5 6 7 8 9
IgG
αCTGF

Figure 7

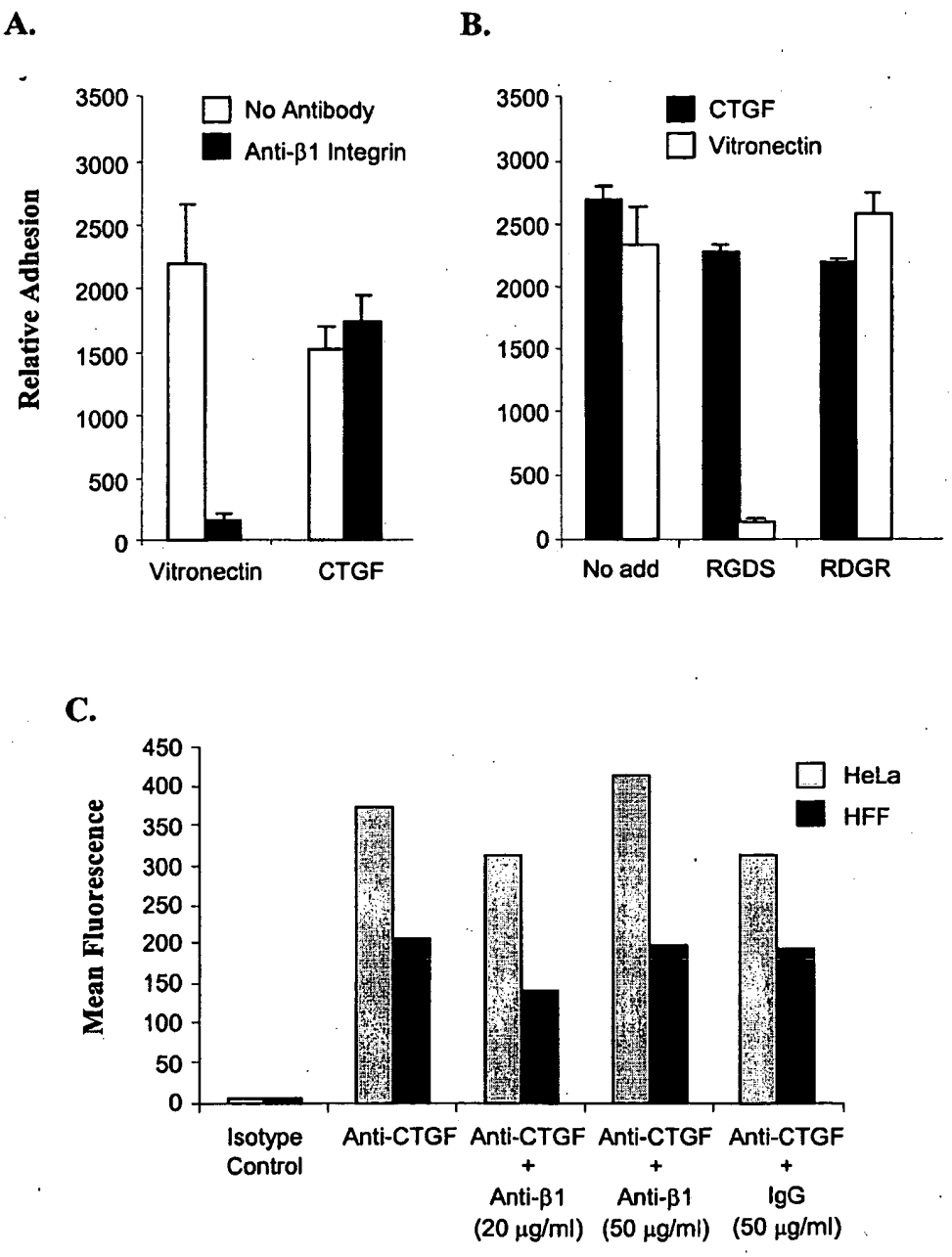

A.
B.
C.
Relative Adhesion
3500
3000
2500
2000
1500
1000
500
0
No Antibody
Anti-β1 Integrin
Vitronectin
CTGF
CTGF
Vitronectin
No add
RGDS
RDGR
Mean Fluorescence
450
400
350
300
250
200
150
100
50
0
HeLa
HFF
Isotype Control
Anti-CTGF
Anti-CTGF + Anti-β1 (20 μg/ml)
Anti-CTGF + Anti-β1 (50 μg/ml)
Anti-CTGF + IgG (50 μg/ml)

Figure 8

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/001275

International filing date: 14 January 2005 (14.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/537,053
Filing date: 16 January 2004 (16.01.2004)

Date of receipt at the International Bureau: 03 March 2005 (03.03.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

1287450

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

February 22, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/537,053*
FILING DATE: *January 16, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/01275*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

I hereby certify that this Provisional Application is being deposited as Express Mail (Express Mailing Label No. EV 306 725 332 US with the U.S. Postal Service in an envelope addressed to: Mail Stop Provisional, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on 16 January 2004

Carolyn C. Caires

Carolyn C. Caires

PTO/SB/16 (10-01)
Approved for use through10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mailing Label No. EV 306 725 332 US

APPLICANT(S)/INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Stephen J. David Y.	Klaus Liu	San Francisco, California Palo Alto, California

☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

CONNECTIVE TISSUE GROWTH FACTOR SIGNALING

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number ____ → *Place Customer Number Bar Code Label here*

OR *Type Customer Number here*

☒ Firm or Individual Name	Christopher Turner, Ph.D.				
Company	FibroGen, Inc.	Dept.	Intellectual Property Dept.		
Address	225 Gateway Blvd.				
City	South San Francisco	State	CA	ZIP	94080
Country	US	Telephone	650-866-7200	Fax	650-866-7292

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 17
☒ Drawing(s) *Number of Sheets* 8
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☐ Other (specify)

METHOD OF PAYMENT OF FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the fees
☒ The Commissioner is hereby authorized to charge any fee due or credit any overpayment to Deposit Account Number: 50-0811
☐ Payment by credit card. Form PTO-2038 is attached.

FEE AMOUNT ($) 80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME Christopher Turner, Ph.D.

TELEPHONE 650-866-7200

Date 16 Jan. 2004

REGISTRATION NO. 45,167

Docket No.: FP0815 P

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

I hereby certify that this Provisional Application is being deposited as Express Mail (Express Mailing Label No. EV 306 725 332 US) with the U.S. Postal Service in an envelope addressed to: Mail Stop Provisional, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on 16 January 2004.

Carolyn C. Caires

Carolyn C. Caires

PTO/SB/17 (01-03)
Approved for use through 04/30/2003. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

FEE TRANSMITTAL for FY 2003

Effective 01/01/2003. Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
U.S. Serial No.	To be assigned
Filing Date	Concurrently herewith
Applicant(s)	Klaus and Liu
Examiner Name	To be assigned
Art Unit	To be assigned
Docket No.	FP0815 P

METHOD OF PAYMENT *(check all that apply)*

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: 50-0811

Deposit Account Name: FibroGen, Inc.

The Commissioner is authorized to: *(check all that apply)*

☒ Charge any fee(s) indicated below ☒ Credit any overpayments

☒ Charge any additional fee(s)

☐ Charge fee(s) indicated below, **except for the filing fee** to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below		Fee Paid
Total Claims		-20** =		X	= 0.00
Independent Claims		- 3** =		X	= 0.00
Multiple Dependent					=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

***or number previously paid, if greater; For Reissues, see above*

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid **SUBTOTAL (3)** ($) 0.00

SUBMITTED BY				(Complete *if applicable*)	
Name (Print/Type)	Christopher Turner, Ph.D.	*Registration No. (Attorney/Agent)*	45,167	*Telephone*	650-866-7200
Signature				*Date*	16 January 2004

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

This collection of information is required by 37 CFR 1.17 and 1.27. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, Washington, DC 20231.**

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

CONNECTIVE TISSUE GROWTH FACTOR SIGNALING

BACKGROUND OF THE INVENTION

Heparan Sulfate Proteoglycans (HSPG)

Heparan sulfate proteoglycans (HSPGs) are components of the extracellular milieu and are classified as either membrane anchored, e.g., glypicans; transmembrane, e.g., syndicans; or cell associated, e.g., perlecan. Additionally, HSPGs include cell membrane proteins such as betaglycan, CD44/epican, and testican. HSPGs consist of a core protein decorated with covalently linked heparan sulfate (HS) chains. (See, e.g., Bernfield et al. (1999) Annu Rev Biochem 68:729-777.) The HS chains are polysaccharides composed of repeating disaccharide units of uronic acid (iduronate or glucuronate) and glucosamine. (Bernfield et al., supra.) The disaccharide units are selectively acetylated at the N position of glucosamine; sulfated at the N, 3-O, and 6-O positions of glucosamine; and/or sulfated at the 2-O position of iduronic acid residues.

HSPGs mediate signaling activities based on the structure and sulfation of their HS chains, which influence interaction with signaling molecules. (See, e.g., Rapraeger (2002) Methods Cell Biol 69:83-109.) For example, specific sulfation of 2-*O* and 6-*O* positions on HS chains is necessary for fibroblast growth factor (FGF) signal transduction. Specifically, the 2-*O* sulfation is required for binding of basic FGF to heparin, and 6-*O* sulfation is required for bFGF dimerization and receptor activation. (Pye et al. (2000) Glycobiology 10:1183-1192; Schlessinger et al. (2000) Mol Cell 6:743-750.) Additional signaling pathways that require HSPGs include Wnt, interferon (IFN)-γ, transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and hepatocyte growth factor. (Reichsman et al. (1996) J. Cell Biol. 135:819-827; Lortat-Jacob et al. (1995) Biochem J 310:497-505; Lyon et al. (1997) J Biol Chem 272:18000-18006; Soker et al. (1994) Biochem Biophys Res Commun 203:1339-1347; and Zioncheck et al. (1995) J Biol Chem 270:16871-16878.)

Sulfation of HS chains is tissue specific, and changes in sulfation have been correlated with regulatory changes in growth factor signaling. (See, e.g., Brickman et al. (1998) J Biol Chem 273:4350-4359; Ai et al. (2003) J Cell Biol 162:341-351.) Mutations that alter HSPG formation, organization, or sulfation lead to defects in signaling pathways. (See, e.g., Forsberg and Kjellen (2001) J Clin Invest 108:175-180; Takei et al. (2004) Development 131:73-82.) Similarly, mutations in enzymes that alter sulfation patterns on HSPGs at the cell surface can lead to modification in cell signaling. (See, e.g., Ai et al., *supra.*)

Connective Tissue Growth Factor (CTGF)

CTGF is a 36 kD, cysteine-rich, heparin-binding, secreted glycoprotein originally isolated from the culture media of human umbilical vein endothelial cells. (Bradham et al. (1991) J Cell Biol 114:1285-1294; Grotendorst and Bradham, USPN 5,408,040.) CTGF belongs to the CCN (CTGF, Cyr61, Nov) family of proteins, which includes the serum-induced immediate early gene product Cyr61, the putative oncogene Nov, the src-inducible gene CEF-10, the Wnt-inducible secreted protein WISP-3, and the anti-proliferative protein HICP/rCOP. (O'Brian et al. (1990) Mol Cell Biol 10:3569-3577; Joliot et al. (1992) Mol Cell Biol 12:10-21; Ryseck et al. (1990) Cell Growth and Diff 2:225-233; Simmons et al. (1989) Proc. Natl. Acad. Sci. USA 86:1178-1182; Pennica et al. (1998) Proc Natl Acad Sci U S A, 95:14717-14722; and Zhang et al. (1998) Mol Cell Biol 18:6131-6141.) CCN proteins are characterized by conservation of 38 cysteine residues that constitute over 10% of the total amino acid content and give rise to a modular structure with N- and C-terminal domains. The modular structure of CTGF includes conserved motifs for insulin-like growth factor binding protein (IGF-BP) and von Willebrand's factor (VWC) in the N-terminal domain, and thrombospondin (TSP1) and a cystine-knot motif in the C-terminal domain.

CTGF expression is induced by members of the Transforming Growth Factor beta (TGFβ) superfamily, which includes TGFβ-1, -2, and -3, bone morphogenetic protein (BMP)-2, and activin, as well as a variety of other regulatory modulators including dexamethasone, thrombin, vascular endothelial growth factor (VEGF), and angiotensin II. (Franklin (1997) Int J Biochem Cell Biol 29:79-89; Wunderlich (2000) Graefes Arch Clin Exp Ophthalmol 238:910-915; Denton and Abraham (2001) Curr Opin Rheumatol 13:505-511; and Riewald (2001) Blood 97:3109-3116.)

Members of the CCN family are expressed upon primary stimulation of a cell, and are thought to modulate subsequent cell signaling events. Although CTGF has been shown to interact with numerous factors including VEGF, TGFβ, insulin-like growth factor (IGF), integrins, and HSPGs, the physiological importance of such interactions is not fully understood. (Inoki et al. (2002) FASEB J 16: 219-221; Abreu et al. (2002) Nat Cell Biol 4: 599-604; Kim et al. (1997) Proc Natl Acad Sci USA 94:12981-12986; Lau and Lam (1999) Exp Cell Res 248:44-57; Gao and Brigstock (2003) J Biol Chem 10.1074/jbc.M313204200.) CTGF expression in healthy tissue is typically low; however, increased expression at both the mRNA and protein level has been correlated with various disorders. One of the strongest correlations exists between CTGF expression and the degree of tissue fibrosis associated with a disorder. (Abraham et al. (2000) J Biol Chem 275:15220-15225; Dammeier et al. (1998) Int J Biochem Cell Biol 30:909-922; diMola et al. (1999) Ann Surg 230(1):63-71; Igarashi et al. (1996) J Invest

Dermatol 106:729-733; Ito et al., supra; Williams et al. (2000) J Hepatol 32:754-761; Clarkson et al. (1999) Curr Opin Nephrol Hypertens 8 :543-548; Gupta et al. (2000) Kidney Int 58:1389-1399; Riser et al. (2000) J Am Soc Nephrol 11:25-38.) CTGF is also expressed at specific times and locations during development and appears to be important in regulating skeletal development. CTGF has also been associated with angiogenesis, potentially regulating both neovascularization during development and angiogenesis associated with wound healing.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, and 1C show dose-dependent adhesion of cells to CTGF presented by epitope-specific anti-CTGF monoclonal antibodies.

Figures 2A, 2B, 2C, and 2D show adhesion of cells to CTGF is dependent on the orientation of CTGF, as defined by epitope-specific anti-CTGF antibodies, and requires CTGF domain 4.

Figures 3A, 3B, and 3C show adhesion of cells to CTGF is dependent on heparan sulfate moieties associated with the adhering cells.

Figure 4 shows binding of CTGF to cells is effectively competed by heparin derivatives containing specific sulfation patterns, but not by derivatives lacking such sulfation.

Figures 5A, 5B, and 5C show adhesion of cells to CTGF can be competed by heparin derivatives containing specific sulfation patterns, but not by derivatives lacking such sulfation.

Figures 6A and 6B show betaglycan directly interacts with CTGF, and betaglycan, TGF-β, and CTGF form a ternary complex associated with cell signaling.

Figure 7 shows CTGF interacts with basic FGF, and that bFGF and betaglycan compete for binding to CTGF.

DESCRIPTION OF THE INVENTION

Before the present compositions and methods are described, it is to be understood that the invention is not limited to the particular methodologies, protocols, cell lines, assays, and reagents described, as these may vary. It is also to be understood that the terminology used herein is intended to describe particular

embodiments of the present invention, and is in no way intended to limit the scope of the present invention as set forth in the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural references unless context clearly dictates otherwise. Thus, for example, a reference to "a fragment" includes a plurality of such fragments, a reference to an "antibody" is a reference to one or more antibodies and to equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications cited herein are incorporated herein by reference in their entirety for the purpose of describing and disclosing the methodologies, reagents, and tools reported in the publications that might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Invention

The present invention provides novel heparan sulfate constructs and compositions involved in binding of connective tissue growth factor (CTGF) to cells and CTGF-mediated cell adhesion. Such heparan sulfate moieties may be present on the surface of a cell or contained within the extracellular matrix, which surrounds cells *in vivo* and *in vitro*. Such heparan sulfate moieties are often present *in vivo* in the form of heparan sulfate proteoglycans (HSPGs). The heparan sulfate moieties may also be present as soluble molecules, e.g., in a form chemically identical or similar to heparin. Such soluble forms are useful as therapeutic agents for use in modulating the association of CTGF with cells, the extracellular matrix, or other components, e.g., growth factors, etc.

The heparan sulfate moieties encompassed in the present invention are generally defined according to their ability to bind CTGF or fragments thereof. Specific fragments of CTGF include the C-terminal half of CTGF, more specifically the domain encoded by exon 5. (See, e.g., International Publication Nos. WO 96/38172 and WO 00/35939.) Additionally, CTGF fragments for use in defining heparan sulfate moieties of the present invention include those described in International Publication No. WO 99/07407; Gao and Brigstock (2003), *supra*; Ball et al. (2003) J Endocrinol 176:R1-7; Ball et al. (1998) Biol Reprod

59:828-835; and Brigstock et al. (1997) J Biol Chem 272:20275-20282; all of which are incorporated by reference herein in their entirety.

In certain aspects, a fragment of CTGF is characterized by the presence of the cystine-knot (CK) domain. Cystine-knot domains are found in various proteins including glycoprotein hormones and extracellular proteins. The C-terminal cystine knot-like domain (CTCK), found in CTGF and several other CCN family members, and other growth factors, e.g., TGFβ, nerve growth factor (NGF), platelet-derived growth factor (PDGF), noggin, and gonadotropin, consists of 2 highly twisted antiparallel pairs of beta-strands containing three disulphide bonds. The domain is non-globular and little is conserved among these presumed homologs except for their cysteine residues. The CT and CTCK domains are predicted to form homodimers. Such proteins containing cystine-knot domains may be used to further characterize heparan sulfate (HS) and/or heparin-like molecules of the invention. Specific molecules may be selected based on selectivity in binding among the various CK-containing proteins; e.g., a molecule may be selected based on its binding to CTGF and other CCN family members, but not other growth factors such as TGF-β, basic FGF (bFGF), etc.; or a molecule may be selected based on its binding to CTGF, but not other CCN family members; etc.

Binding characteristics of any particular HS or heparin-like molecule for use in the present invention can be modified by altering the length, e.g., the number of dissacharide repeats, in the molecule; the charge, e.g., the number of sulfated residues; and/or the charge distribution, e.g., the degree of N-sulfation, 2-*O*-sulfation, and 6-*O*-sulfation on respective sugar residues. Such modifications can be measured for desired characteristics using binding and adhesion assays, as described in the Examples, using CTGF, fragments thereof, and other potential binding proteins, e.g., CK-containing proteins, as described above.

The HS and heparin-like molecules so defined can be utilized to modulate the bioactivity of CTGF. In particular embodiments, the molecule alters CTGF bioactivity by altering the ability of CTGF to interact with a cell surface or an endogenous extracellular matrix-associated HSPG. As other signaling pathways, e.g., bFGF signaling, are known to involve HSPG binding, the present invention specifically provides methods to inhibit the ability of CTGF to interact with HSPG without affecting the activity of other heparin binding growth factors. Such methods comprise administering an HS or heparin-like molecule of the invention to a subject. In these particular embodiments, the molecule is characterized by its ability to inhibit CTGF-mediated cell adhesion or cell binding without affecting the binding or signaling of other factors, e.g., other CCN family members and/or other growth factors such as VEGF or bFGF, as desired.

The present invention also provides specific HSPGs herein identified as CTGF-binding components. In one particular embodiment, the HSPG is betaglycan. As used herein, "betaglycan", also known as "TGF-β type III receptor", is selected from human betaglycan (GenBank Accession No. AAA67061) or an orthologous protein obtained from any other species. (See, e.g., GenBank Accession No. CAB64374; GenBank Accession No. AAC28564; and GenBank Accession No. AAA40813.) Additionally, betaglycan may comprise any fragment of a full-length betaglycan protein, and especially fragments of betaglycan described, e.g., in Lopez-Casillas et al. (1994) J Cell Biol 124(4):557-568; and Pepin et al. (1995) FEBS Lett 377: 368-372; both of which are incorporated by reference herein in their entirety. Further, betaglycan may comprise naturally-occurring or recombinant soluble betaglycan as described, e.g., in Zhang et al. (2001) Immunol Cell Biol 79:291-297; and Vilchis-Landeros et al. (2001) Biochem J 355:215-222, both of which are incorporated by reference herein in their entirety.

Betaglycan is a 349 amino acid transmembrane glycoprotein with a large extracellular region, which binds TGF-β, and a small cytoplasmic region. Betaglycan is considered an "accessory" receptor, since it appears to regulate the interaction of TGF-β with the signaling receptors, TGF-β type I receptor and TGF-β type II receptor, and thus regulate cell stimulation by TGF-β. (See, e.g., López-Casillas et al. (1993) Cell 73:1435-1444; Sankar et al. (1995) J Biol Chem 270:13567-13572; Lastres et al. (1996) J Cell Biol 133:1109-1121; and Sun and Chen (1997) J Biol Chem 272:25367-25372.) The extracellular domain of betaglycan contains heparan and chondroitin sulphate chains; however, it is thought to be the core protein that binds TGF-β isoforms.

The present invention provides methods to modulate growth factor activity mediated by CTGF. For example, the present examples demonstrate that CTGF and TGF-β form a physical complex with betaglycan. As betaglycan is required for proper cell stimulation by TGF-β, in particular embodiments the present invention provides methods to alter TGF-β signaling by inhibiting CTGF interaction with cell surface HSPGs. In certain embodiments, the HSPG is betaglycan.

Further, the present examples demonstrate a novel interaction between CTGF and bFGF, and interactions between CTGF and betaglycan are modulated in the presence of bFGF. In particular embodiments, the invention provides methods to modulate CTGF signaling in conjunction with or mediated by bFGF by blocking the capacity of CTGF to interact with cell surface HSPGs. In certain embodiments, the HSPG is betaglycan.

As described above, members of the CCN family share the domain on CTGF responsible for HSPG interaction. Although the specificity between individual members of the CCN family and respective HSPG moieties may vary, a certain degree of similarity would be expected. The invention, by providing means to identify and distinguish between HS or heparin-like molecules specific for CTGF, and HS or heparin-like molecules generally active against CCN family binding, provides methods that can be used to modulate various CCN family signaling pathways. Therefore, in some embodiments, the invention provides methods to modulate the ability of CTGF to alter signaling by blocking the capacity of CCN family members to interact with cell surface HSPGs. In particular embodiments, the method modulates signaling by Wnt, a developmental and oncogenic factor modulated by CCN family proteins, e.g., Wisp-3. In certain embodiments, the HSPG is associated with activity of the LDL receptor-related protein (LRP).

Recently, it has been demonstrated that betaglycan also binds and regulates the actions of other members of the TGF-β superfamily. For example, betaglycan forms a complex with the type II activin receptor. This complex then binds inhibin A and prevents formation of functional activin type I/II receptor complexes. (See. e.g., Lewis et al. (2000) Nature 404:411–414.) The interaction between inhibin and betaglycan also prevents bone morphogenetic protein (BMP), e.g., BMP-2, BMP-7, and BMP-9, signaling. (See, e.g., Wiater and Vale (2003) J Biol Chem 278:7934-7941.) As CTGF interacts with betaglycan and forms ternary complexes with betaglycan and TGF-β, CTGF may also regulate other facets of betaglycan function. In any case, modifying interactions between betaglycan and signaling factors, e.g., inhibin, using methods of the invention is specifically contemplated. In specific aspects, the invention provides methods to modulate the ability of CTGF to alter activin signaling by blocking the capacity of CTGF to interact with cell surface HSPGs. In other aspects, the invention provides methods to modulate the ability of CTGF to alter inhibin activity by blocking the capacity of CTGF to interact with cell surface HSPGs. In still other aspects, the invention provides methods to modulate the ability of CTGF to alter BMP signaling by blocking the capacity of CTGF to interact with cell surface HSPGs. In particular embodiments, the HSPG is betaglycan.

In all of the embodiments described above, it is a specific aspect of the invention that the degree of inhibition in CTGF binding can be regulated using specific HS or heparin-like molecules. As CTGF has been implicated in pathways that may not involve heparan sulfate, it is envisioned that specific pathways may not be affected by the present procedures. For example, CTGF has been show to interact with integrins, a family of cell adhesion receptors. The present invention contemplates modulation of certain CTGF bioactivities, such as those associated with TGF-β signaling, by altering the ability of CTGF to

interact with cell surface or extracellular matrix-associated HSPGs, without affecting or being affected by, e.g., integrin signaling.

EXAMPLES

The invention will be further understood by reference to the following examples, which are intended to be purely exemplary of the invention. These examples are provided solely to illustrate the claimed invention. The present invention is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only. Any methods that are functionally equivalent are within the scope of the invention. Various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Example 1. Production of recombinant human CTGF (rhCTGF)

A recombinant human CTGF baculovirus construct was produced as described in Segarini et al. (2001, J Biol Chem 276:40659-40667). Briefly, a CTGF cDNA comprising only the open reading frame was generated by PCR using DB60R32 (Bradham et al. (1991) J Cell Biol 114:1285-94) as template and the primers 5' gctccgcccgcagtgggatccATGaccgccgcc 3' and 5' ggatccggatccTCAtgccatgtctccgta 3', which add BamHI restriction enzyme sites to the ends of the amplified product. The native start and stop codons are indicated in capital letters.

The resulting amplified DNA fragment was digested with BamHI, purified by electrophoresis on an agarose gel, and subcloned directly into the BamHI site of the baculovirus PFASTBAC1 expression plasmid (Invitrogen Corp., Carlsbad CA). The sequence and orientation of the expression cassette was verified by DNA sequencing. The resulting CTGF expression cassette was then transferred to bacmid DNA by site-specific recombination in bacteria. This bacmid was then used to generate a fully recombinant CTGF baculovirus in Spodoptera frugiperda SF9 insect cells according to protocols supplied by the manufacturer (BAC-TO-BAC Expression System manual; Invitrogen). Expansion of recombinant baculovirus titers in Sf9 insect cells was performed using standard procedures known in the art.

Hi5 insect cells were adapted for suspension growth by serial passage of cells in shake flask culture accompanied by enrichment at each passage for separated cells. Suspension Hi5 cells were cultured in 1L SF900II SFM media (Invitrogen) supplemented with 20 μg/ml gentamicin (Mediatech, Inc., Herndon VA) and 1x lipid (Invitrogen) in disposable 2.8L Fernbach culture flasks (Corning Inc., Acton MA) on a shaker platform at 110 rpm at 27°C. Once cells reached a density of 1.0-1.5x10^6 cells/ml with a viability

of >95%, they were infected with recombinant baculovirus at a multiplicity of infection (MOI) of 10. The cultures were then incubated at 27°C for an additional 40 to 44 hours. The conditioned media, which contains rhCTGF, was collected, chilled on ice, and centrifuged at 5000 x *g*. The supernatant was then passed through a 0.45 mm filter.

Four liters of conditioned media was loaded over a 5 ml HI-TRAP heparin column (Amersham Biosciences Corp., Piscataway NJ) pre-equilibrated with 50 mM Tris (pH7.5),150 mM NaCl. The column was washed with 10 column volumes of 350 mM NaCl, 50mM Tris (pH 7.5). CTGF was eluted from the column with an increasing NaCl salt gradient. Eluted fractions were screened by SDS-PAGE, and those containing CTGF were pooled.

Heparin purified CTGF was diluted to a final conductivity of 5.7 mS with non-pyrogenic double-distilled water and the pH was adjusted to 8.0. A Q-SEPHAROSE strong anion exchange column (Amersham Biosciences) containing approximately 23 ml resin connected in tandem with a carboxymethyl (CM) POROS polystyrene column (Applied Biosystems) containing approximately 7 ml resin was utilized for endotoxin removal, and capture and elution of purified rhCTGF. Prior to the sample load, the tandem column was washed with 0.5 M NaOH, followed by 0.1 M NaOH, and finally equilibration buffer. The load sample was passed over the tandem column, the Q-Sepharose column was removed, and CTGF was eluted from the CM POROS column (Applied Biosystems) with an increasing 350 mM to 1200 mM NaCl gradient. The purity of the eluted fractions containing CTGF were evaluated by SDS-PAGE analysis before forming a final sample pool.

Example 2. Anti-CTGF Monoclonal Antibodies

2.1 *Antibody Production*

Fully human monoclonal antibodies to human CTGF were prepared using HUMAB mouse strains HCo7, HCo12 and HCo7+HCo12 (Medarex, Inc., Princeton NJ). Mice were immunized by up to 10 intraperitoneal (IP) or subcutaneous (Sc) injections of 25-50 mg recombinant human CTGF in complete Freund's adjuvant over a 2-4 week period. The immune response was monitored by retroorbital bleeds. Plasma was screened by ELISA (as described below), and mice with sufficient titers of anti-CTGF immunogolobulin were used for fusions. Mice were boosted intravenously with antigen 3 and 2 days before sacrifice and removal of the spleen.

Single cell suspensions of splenic lymphocytes from immunized mice were fused to one-fourth the number of P3X63-Ag8.653 nonsecreting mouse myeloma cells (American Type Culture Collection (ATCC), Manassas VA) with 50% PEG (Sigma, St. Louis MO). Cells were plated at approximately $1x10^5$ cells/well in flat bottom microtiter plate and incubated for about two weeks in high-glucose DMEM (Mediatech, Herndon VA) containing L-glutamine and sodium pyruvate, 10% fetal bovine serum, 10% P388D1 (ATCC) conditioned medium, 3-5% ORIGEN hybridoma cloning factor (Igen International, Gaithersburg MD), 5 mM HEPES, 0.055 mM 2-mercaptoethanol, 50 mg/ml gentamycin, and 1x HAT (Sigma). After 1-2 weeks, cells were cultured in medium in which the HAT was replaced with HT. Individual wells were then screened by ELISA (described below). Antibody secreting hybridomas were replated, screened again, and, if still positive for anti-CTGF antibodies, were subcloned at least twice by limiting dilution. The stable subclones were then cultured in vitro to generate small amounts of antibody in tissue culture medium for characterization. One clone from each hybridoma that retained the reactivity of the parent cells was used to generate 5-10 vial cell banks stored in liquid nitrogen.

ELISA assays were performed as described by Fishwild et al. (1996, Nature Biotech 14:845-851). Briefly, microtiter plates were coated with 1-2 µg/ml purified recombinant CTGF in PBS at 50 µl/well, incubated at 4°C overnight, then blocked with 200 µl/well 5% chicken serum in PBS/Tween (0.05%). Dilutions of plasma from CTGF-immunized mice or hybridoma culture supernatants were added to each well and incubated for 1-2 hours at ambient temperature. The plates were washed with PBS/Tween and then incubated with a goat-anti-human IgG F_c polyclonal antibody conjugated with horseradish peroxidase (HRP) for 1 hour at room temperature. After washing, the plates were developed with 0.22 mg/ml ABTS substrate (Sigma) and analyzed by spectrophotometer at 415-495 nm.

2.2 *Antibody characterization*

Epitope mapping of antibodies by competitive binding experiments is well known by those skilled in the field of immunology. (See, e.g., Van Der Geld et al. (1999) Clinical and Experimental Immunology 118:487-96.) Each antibody population isolated from cells propagated from a unique cloned hybridoma cell was mapped and assigned to a specific binding domain on human CTGF using standard binding and blocking experiments. (See, e.g., Antibodies: A Laboratory Manual (1988) Harlow and Lane (eds), Cold Spring Harbor Laboratory Press; Tietz Textbook of Clinical Chemistry, 2nd ed., (1994) Chapter 10 (Immunochemical Techniques), Saunders; and Clinical Chemistry: Theory, Analysis, Correlation (1984) Chapter 10 (Immunochemical Techniques) and Chapter 11 (Competitive Binding Assays), C.V. Mosby, St. Louis.) For example, epitope mapping was performed by ELISA analysis using specific recombinantly expressed fragments of CTGF. Antibodies that recognized epitopes, e.g., on the N-

terminal domain of CTGF were identified by ELISA analysis against immobilized fragments obtained from recombinant expression of exon 2 and/or exon 3 of the CTGF gene. Antibodies that specifically recognize N-terminal domains or N-terminal fragments of CTGF (e.g., anti-N1, an antibody having specificity for an N-terminal fragment epitope containing CTGF domain 1; anti-N2, an antibody having specificity for an N-terminal fragment epitope containing CTGF domain 2; etc.) or C-terminal domains or C-terminal fragments of CTGF (e.g., anti-C1, an antibody having specificity for a C-terminal fragment epitope containing CTGF domain 3; anti-C2, an antibody having specificity for a C-terminal fragment epitope containing CTGF domain 4; etc.) were selected and utilized in the following examples.

Example 3. Assays

3.1 ***Cell Adhesion Assay***

Methods for measuring cell adhesion mediated by CTGF are generally known to those skilled in the art. (See, e.g., Babic et al. (1999) Mol Cell Biol 19:2958-296; Ball et al. (2003) J Endocrinol 176:R1-7.) Such methods typically involve application of CTGF directly to plastic tissue culture plates. Unsaturated protein binding capacity is blocked, e.g., with bovine serum albumin, and wells are seeded with cells. Additional factors, e.g., chelators such as EDTA, peptides, organic compounds, antibodies, etc., may be incubated with the cells prior to plating or added concurrently with the cells. Plates are incubated for a suitable length of time, e.g., 30 to 60 min, at a suitable temperature, e.g., 25 to 37°C, to allow cells to adhere; wells are then washed, and adherent cells are measured. Cell measurements may be made by any method known in the art; e.g., cells may be fixed with formalin, stained, e.g., with methylene blue, and quantified by dye extraction and measurement of absorbance, e.g., at 620 nm.

In a preferred method, tissue culture plates are coated with CTGF indirectly using epitope-specific capture antibodies. In the present examples, cells of a tissue culture plate were coated with a human monoclonal antibody specific for human CTGF, and then were blocked with bovine serum albumin to prevent non-specific binding. Cells were added at a seed density of approximately 8×10^4 cells/well. Additionally, either rhCTGF or fragments thereof, or a vehicle control was added to each well, and the plates were incubated for 45 minutes at 37°C. Wells were then washed, and the number of cells retained in each well was measured using a CYQUANT cell proliferation assay kit (Molecular Probes, Inc., Eugene OR).

In experiments using human dermal foreskin fibroblast cells and a human monoclonal antibody specific for human CTGF domain 3 (anti-C1), dose-sensitive cell adhesion was seen when any of the parameters,

i.e., amount of CTGF, anti-CTGF antibody, or cell number, was altered while the remaining parameters were held constant. For example, a dose-sensitive increase in the number of cells retained in each well was seen when either antibody concentration was held constant (10 μg/ml) and CTGF concentration was increased (Figure 1A), or when CTGF concentration was held constant (2 μg/ml) and anti-CTGF antibody concentration was increased (Figure 1B). Similarly, a dose-sensitive increase in the number of cells retained in each well was seen when cells were titrated in wells coated with a constant amount of antibody (10 μg/ml) and CTGF (2 μg/ml) (Figure 1C).

3.2 *CTGF Binding Assay*

Methods for measuring binding of CTGF to cells are generally known to those skilled in the art. (See, e.g., Nishida (1998) Biochem Biophys Res Commun 247:905-909; Segarini et al. (2001) J Biol Chem 276:40659-40667.) Such methods typically involve labeling CTGF with a detectable moiety, e.g., a radioactive or fluorescent tag, applying the labeled CTGF to cells, washing the cells to remove unbound CTGF, and then measuring the amount of label that remains associated with the cells. Cells may be attached to a surface, e.g., a tissue culture plate, or in suspension. Labeling cells in suspension allows analysis by flow cytometry, e.g. using fluorescently labeled CTGF and a fluorescent-activated cell sorter (FACS).

In a preferred method, cells were suspended in media containing CTGF under conditions suitable for binding of CTGF to cellular targets. Cells may optionally be treated prior to or concurrently with CTGF exposure; for example, cells may be treated with enzymes that alter cell surface moieties, molecules that compete competitively or non-competitively with CTGF for binding to cells, etc. Following incubation to allow CTGF to bind to cells, cells were washed and then incubated with fluorescently-labeled anti-CTGF antibody. The level of CTGF binding was then measured using a FACS apparatus.

3.3 *Co-immunoprecipitation Assay*

Co-immunoprecipitation is a purification procedure used to determine if two different molecules, e.g., proteins, directly interact. Basically, an antibody specific to a protein of interest is added to a cell lysis under conditions suitable for antibody binding to the protein. The antibody-protein complex is then collected, e.g., using protein-G sepharose, which binds most antibodies. Any molecules that are bound to the precipitated protein will also be collected. Identification of proteins can be determined by, e.g., western blot or by direct sequencing of the purified protein(s). Several commercial kits, e.g., the PROFOUND co-immunoprecipitation kit from Pierce Biotechnology, Inc. (Rockford IL) are also available.

In the present examples, co-immunoprecipitations were performed as follows. The surface of intact cells was iodinated with ^{125}I prior to lysing cells and fractionating on a CTGF affinity column. Alternatively, CTGF and labeled cells were incubated for a period sufficient for CTGF binding to cells, and then cells were lysed and immunoprecipitations were performed using anti-CTGF specific antibodies. Antibody complexes were collected from the lysate using protein-G sepharose, and pelleted by centrifugation. Proteins eluted from affinity columns or collected by immunoprecipitation were analyzed by fractionation on SDS-PAGE and visualized by autoradiography. In similar experiments, unlabeled cells or specific proteins were mixed with CTGF alone or in the presence of additional factors, and immunoprecipitations were performed. Following fractionation, proteins were transferred to membranes and probed by western analysis.

Example 4. Regions of CTGF Involved in Cell Binding and Adhesion

4.1 ***Various cell types utilize a similar mechanism in CTGF-mediated adhesion***

The cell adhesion assay described in Example 3.1 was used to identify regions of CTGF involved in cell adhesion. Wells of a tissue culture plate were coated with monoclonal antibodies specific for either amino-terminal domains (anti-N1 or anti-N2 antibodies) or carboxy-terminal domains (anti-C1, -C2) of CTGF (see Figure 2A). HFF were seeded into wells and adhesion was measured as described in Example 3.1.

As shown in Figures 2B, antibodies specific for epitopes associated with the C-terminal domain of CTGF presented CTGF to cells in a manner that facilitated cell adhesion. However, antibodies specific for epitopes on the N-terminal domain of CTGF did not orient CTGF in a manner that allowed cell adhesion.

4.2 ***The C-terminal half of CTGF mediates cell adhesion***

To further define the region of CTGF responsible for cell adhesion, the procedure used in Example 4.1 was further modified as follows. Wells of a tissue culture plate were coated with monoclonal antibodies specific for either amino-terminal (anti-N1) or carboxy-terminal (anti-C1) domains of CTGF (see Figure 2A). Wells were then seeded with HFF in the presence of no CTGF, full-length CTGF, the N-terminal half of CTGF (NH2 fragment), or the C-terminal half of CTGF (COOH fragment). Wells were incubated and adhesion was measured as described in Example 3.1.

Consistent with the results shown in Example 4.1, presentation of full-length CTGF using anti-C1 antibodies facilitated cell adhesion, whereas presentation using anti-N1 antibodies did not (Figure 2C). Further, the C-terminal half of CTGF, when captured using anti-C1 antibodies, was sufficient to provide cell adhesion equivalent to adhesion provided by full-length CTGF. Additionally, the binding was dose responsive, increasing with increasing amounts of CTGF or CTGF fragment. The N-terminal half of CTGF, however, did not provide a suitable substrate for cell adhesion (Figure 2C). The data show that the C- terminal half of CTGF mediates CTGF-dependent adhesion.

4.3 *CTGF-dependent adhesion requires domain 4*

The cell adhesion assay described in Example 3.1 was used to further define the portion of the C-terminal half of CTGF involved in cell adhesion. Wells of a tissue culture plate were coated with monoclonal antibodies specific for the "hinge" domain of CTGF (anti-H1 antibodies) (see Figure 2A). Wells were then seeded with HFF (8×10^4 cells/well) in the presence of no CTGF, full-length CTGF, or a CTGF construct lacking domain 4 (CTGFΔ4). Wells were incubated and adhesion was measured as described in Example 3.1.

Although HFF were able to adhere to full-length CTGF, they were not able to bind to CTGF lacking domain 4 (CTGFΔ4) (Figure 2D). This result suggests that domain 4, which contains the cystine knot (CK) motif, is necessary for CTGF-mediated cell adhesion.

Example 5. HSPGs are Required for CTGF Binding and CTGF-mediated Adhesion

5.1 *Heparan sulfate is involved in CTGF binding and CTGF-dependent cell adhesion*

CTGF has been described as a heparin-binding growth factor. As cells may carry a variety of proteoglycan moieties on their surface, e.g., heparan sulfate, chondroitin sulfate, etc. (see Figure 3A), the following experiment was conducted to determine the specificity of CTGF for such moieties. The cell adhesion and cell binding assays were conducted as described in Examples 3.1 and 3.2, respectively, except prior to seeding cells were treated for 1 hour at 37°C with either vehicle, 4 units/ml heparinase I, or 2 units/ml chontroitinase ABC.

As shown in Figure 3B, CTGF-dependent cell adhesion was inhibited by pretreatment of cells with heparinase, but not chondroitinase.

To further examine the requirement for heparan sulfate proteoglycans in CTGF-mediated cell adhesion, adhesion was measured in the presence of increasing amounts of heparin. Heparin and heparan sulphate both consist of repeating disaccharides of uronic acid and glucosamine, but the proportion of N-sulfation of heparan sulfate is typically below 50%, while sulfation of heparin is usually 70% or higher. The cell adhesion assay was conducted as described in Examples 3.1, except increasing concentrations of low molecular weight heparin (LMWH) was additionally added to each adhesion reaction.

As shown in Figure 3C, CTGF-dependent adhesion was inhibited by soluble heparin in a concentration-dependent manner. This result supports the conclusion that CTGF-mediated cell adhesion requires heparan sulfate moieties, i.e., HSPGs.

5.2 ***Differential inhibition of CTGF-mediated cell adhesion and cell binding by modified heparin sulfate oligomers***

The sulfate groups of heparin include 2-O-sulfation of iduronate residues, 6-O-sulfation of iduronate residues, and amino group sulfation (N-sulfation) of glucosamine residues. Sulfates can be selectively removed using chemical methods known to those skilled in the art. Such methods, as described below, can be applied either solely or jointly to obtain a polysaccharide derivative with a desired sulfation pattern. Oligosaccharide libraries can be obtained and screened using methods known to those skilled in the art. (See, e.g., Jemth et al. (2003) J Biol Chem 278: 24371-24376; and Ashikari-Hada et al. (2004) J Biol Chem 10.1074/jbc.M313523200.)

Both O- and N-sulfate groups can be removed, e.g., by heating a pyridinium salt of heparin at 80°C for four hours in dimethylsulfoxide. (See, e.g., Nagasawa et al. (1977) Carbohydr Res 58:47-55.) Since the elimination rate of the N-sulfate group is much greater than that of the O-sulfate group, carrying out the reaction under mild conditions, e.g., reaction at or below 20°C, produces selective de-N-sulfation. (See, e.g., Inoue and Nagasawa (1976) Carbohydr Res 46:87-95.) Sulfate groups can be removed from ether (O-sulfation) linkages under strongly alkaline conditions. The resulting epoxide rings can then be cleaved to yield primarily iduronate residues. Removal of 6-O-sulfation can be carried out, e.g., as described in Takano et al. (1998, Carbohydr Lett 3:71-77).

To determine the specificity of sulfation and charge distribution for CTGF-mediated cell adhesion and cell binding, experiments as described in Examples 3.1 and 3.2, respectively, were performed with the following modification. Combination of HFF cells with CTGF was accompanied by addition of

increasing concentrations of soluble LMWH or heparin sulfate oligomers that were modified to contain differing amounts of sulfation and acetylation covalently bound to either oxygen (O) or nitrogen (N).

As shown in Figure 4, binding of CTGF to HFF requires specific sulfation of heparan sulfate or heparin-like molecules. Specifically, heparin and oversulfated derivatives thereof substantially inhibit CTGF binding to cells. However, de-O-sulfated heparin derivatives were less effective at inhibiting binding, and de-N-sulfation showed no inhibitory capacity. Thus, cell binding by CTGF requires N-sulfation, and is further augmented by both 2-O- and 6-O-sulfation. The dashed line in Figure 4 indicates the level of CTGF binding without any addition of heparin or derivatives. Figures 5A, 5B, and 5C, which show the effect of soluble LMWH or modified heparin sulfate oligomers on CTGF-mediated cell adhesion, confirm the effect of desulfation seen in the CTGF binding assay above.

The data show that there are specific modifications on heparin sulfate that are critical for CTGF binding and cell adhesion, whereas other modifications do not affect CTGF binding or responsiveness. Specifically, the data point to the importance of N-sulfation and O-sulfation of heparin sulfate proteoglycans as being critical for CTGF binding and signaling. These modifications are unique to CTGF and different from modifications known to mediate signaling of other heparin binding growth factors, such as, e.g., bFGF or PDGF. Thus, specific therapeutics can be derived based on heparan sulfate or heparin-like molecules which specifically inhibit CTGF function but do not inhibit the bioactivity of other heparin binding growth factors.

Example 6. Betaglycan is a CTGF-binding HSPG

6.1 *CTGF binds directly to betaglycan*

Identification of cell receptors for CTGF was carried out using co-immunoprecipitation procedures as described in Example 3.3. Initial experiments using radiolabeled cells identified betaglycan as a primary CTGF-binding protein on the cell surface (data not shown). Subsequent experiments using soluble betaglycan (sBetaglycan) demonstrated dose-sensitive interaction between betaglycan and CTGF (Figure 6A). Together, this data shows that betaglycan is a cell surface HSPG that functions as a specific receptor for CTGF.

6.2 *CTGF binds TGFβ and betaglycan in a ternary complex in an HSPG-dependent fashion*

Betaglycan is also known as TGF-β type III receptor and has been shown to facilitate cell stimulation by TGF-β. CTGF has also been associated with TGF-β signaling as an immediate early response factor

produced by cells upon TGF-β signaling. To determine the functional nature of possible interactions between betaglycan, CTGF, and TGF-β, immunoprecipitations were performed as follows. Soluble betaglycan, [^{125}I]-labeled TGF-β, and CTGF were mixed under conditions suitable for interaction, and then complexes were isolated using anti-CTGF antibodies bound to a solid bead matrix. The data show that CTGF, betaglycan and TGF-β form a ternary complex that is dependent on the heparin binding potential of CTGF (Figure 6B). The present invention contemplates that inhibition of ternary complex formation may inhibit betaglycan-dependent CTGF signaling, and may thereby modify TGF-β signaling.

6.3 ***CTGF binds FGF and betaglycan in a ternary complex in an HSPG dependent fashion***

Fibroblast growth factors bind to HSPGs, and signaling by basic and acidic FGF requires this interaction. To determine if the HSPG-dependent interaction between CTGF and betaglycan involves or is modified by FGF, immuno-precipitations were performed as follows. Soluble betaglycan, bFGF, and CTGF were mixed under conditions suitable for interaction, and then complexes were isolated using anti-CTGF antibodies bound to a solid bead matrix. As shown in Figure 7, binding between CTGF and betaglycan is adversely influenced by bFGF in a dose-sensitive manner. Surprisingly, the interaction was not due solely to competition between CTGF and bFGF to heparan sulfate moieties on betaglycan. There was also a clear interaction between CTGF and bFGF, as immunoprecipitation of CTGF in the presence of bFGF, without betaglycan, demonstrated clear interaction between the two growth factors. The result shows that a novel interaction between CTGF and bFGF has been identified, and that selective inhibition of ternary complex formation may inhibit CTGF signaling alone, coordinated signaling between CTGF and TGF-β, and/or coordinated or independent signaling by bFGF.

Various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein are hereby incorporated by reference in their entirety.

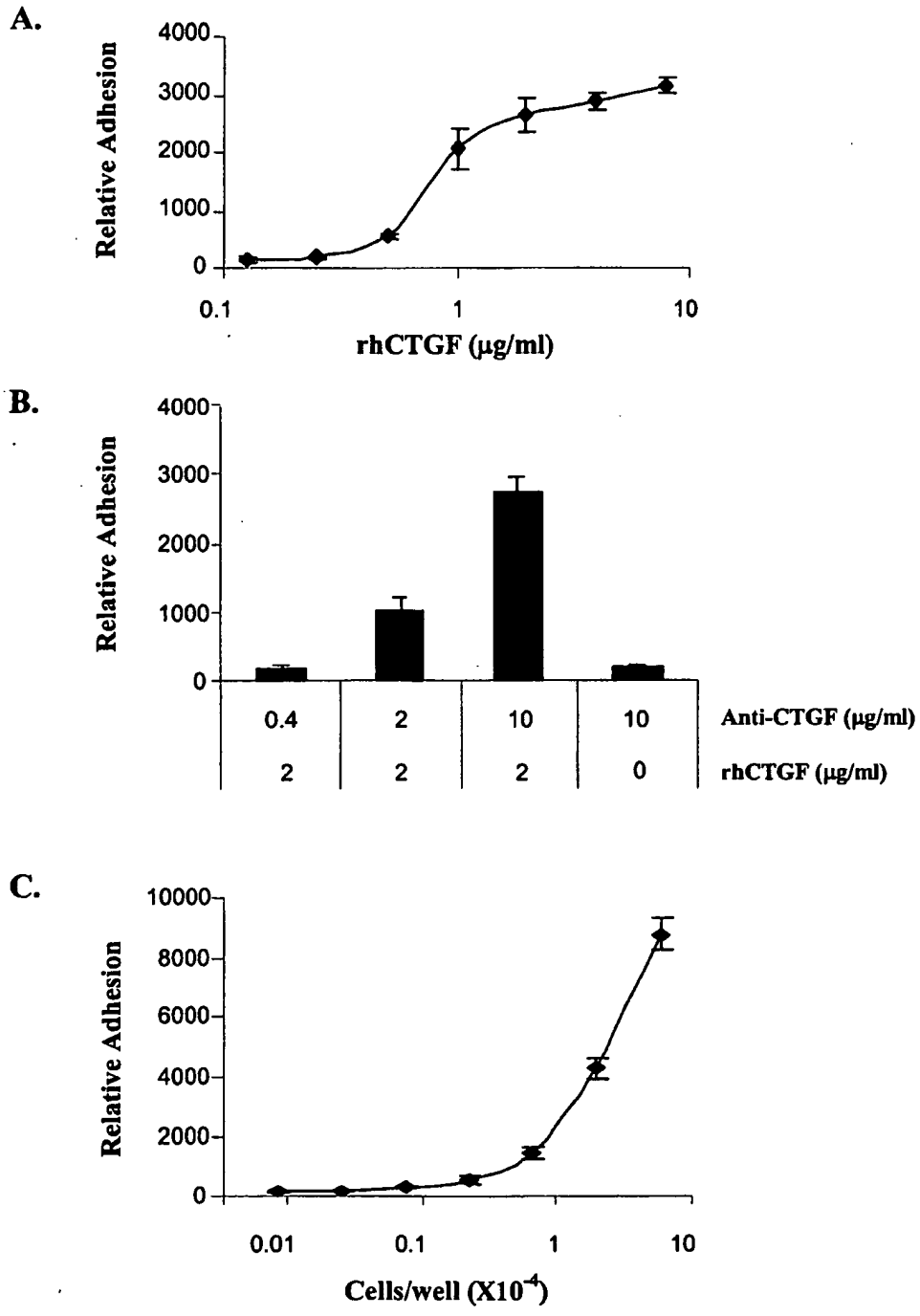

A.
Relative Adhesion
4000
3000
2000
1000
0
0.1
1
10
rhCTGF (μg/ml)
B.
Relative Adhesion
4000
3000
2000
1000
0
0.4
2
10
10
Anti-CTGF (μg/ml)
2
2
2
0
rhCTGF (μg/ml)
C.
Relative Adhesion
10000
8000
6000
4000
2000
0
0.01
0.1
1
10
Cells/well (X10-4)

Figure 1

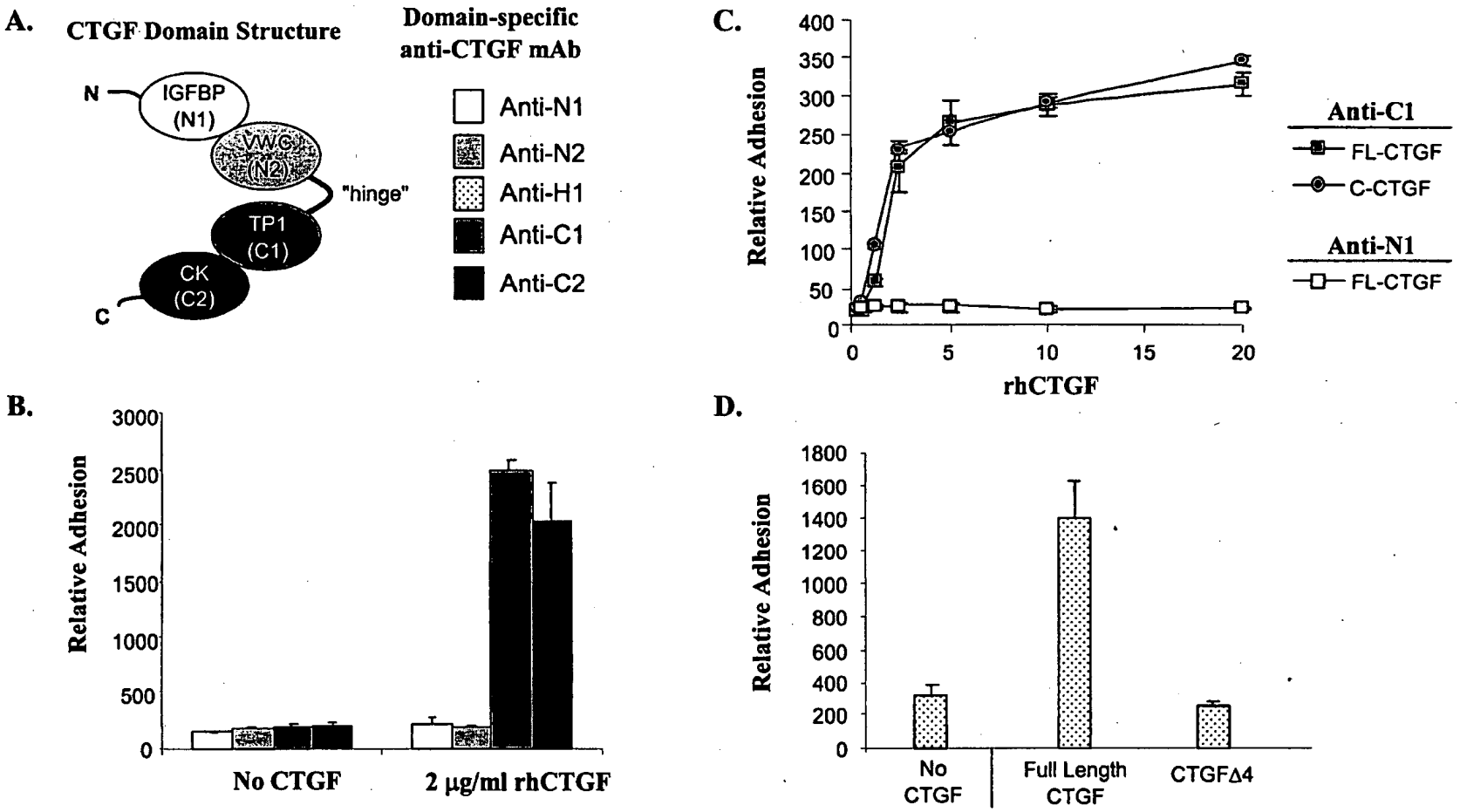
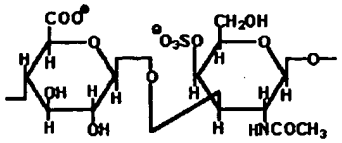
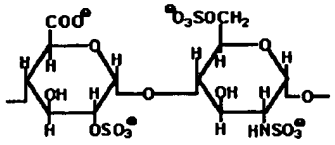

A.
CTGF Domain Structure
Domain-specific anti-CTGF mAb
N
IGFBP (N1)
VWC (N2)
"hinge"
TP1 (C1)
CK (C2)
C
Anti-N1
Anti-N2
Anti-H1
Anti-C1
Anti-C2
B.
Relative Adhesion
3000
2500
2000
1500
1000
500
0
No CTGF
2 µg/ml rhCTGF
C.
Relative Adhesion
400
350
300
250
200
150
100
50
0
0
5
10
15
20
rhCTGF
Anti-C1
FL-CTGF
C-CTGF
Anti-N1
FL-CTGF
D.
Relative Adhesion
1800
1600
1400
1200
1000
800
600
400
200
0
No CTGF
Full Length CTGF
CTGFΔ4

Figure 2

A.

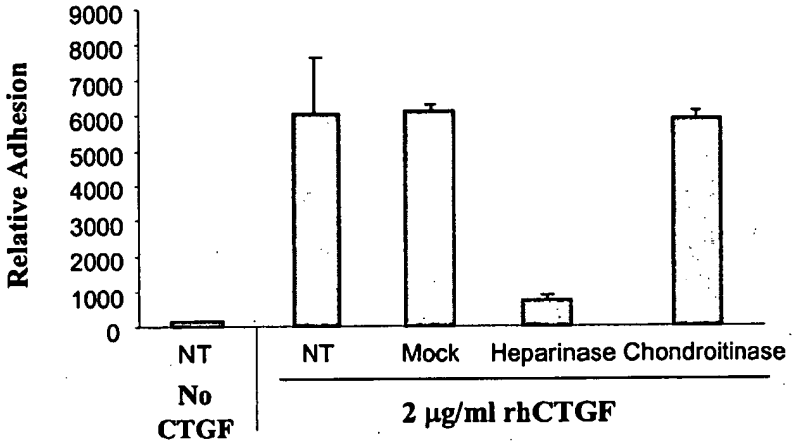


Chondroitin sulfate:
Glucuronate-glucosamine
β (1,3) linkage



Heparan sulfates:
Glucuronate-glucosamine or
Iduronate-glucosamine
α (1,4) linkage

B.



C.

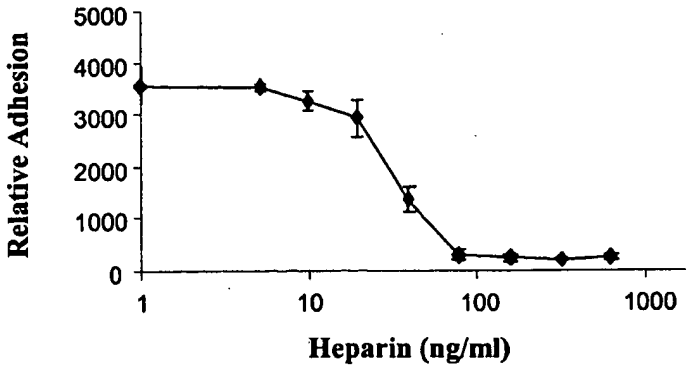


Figure 3

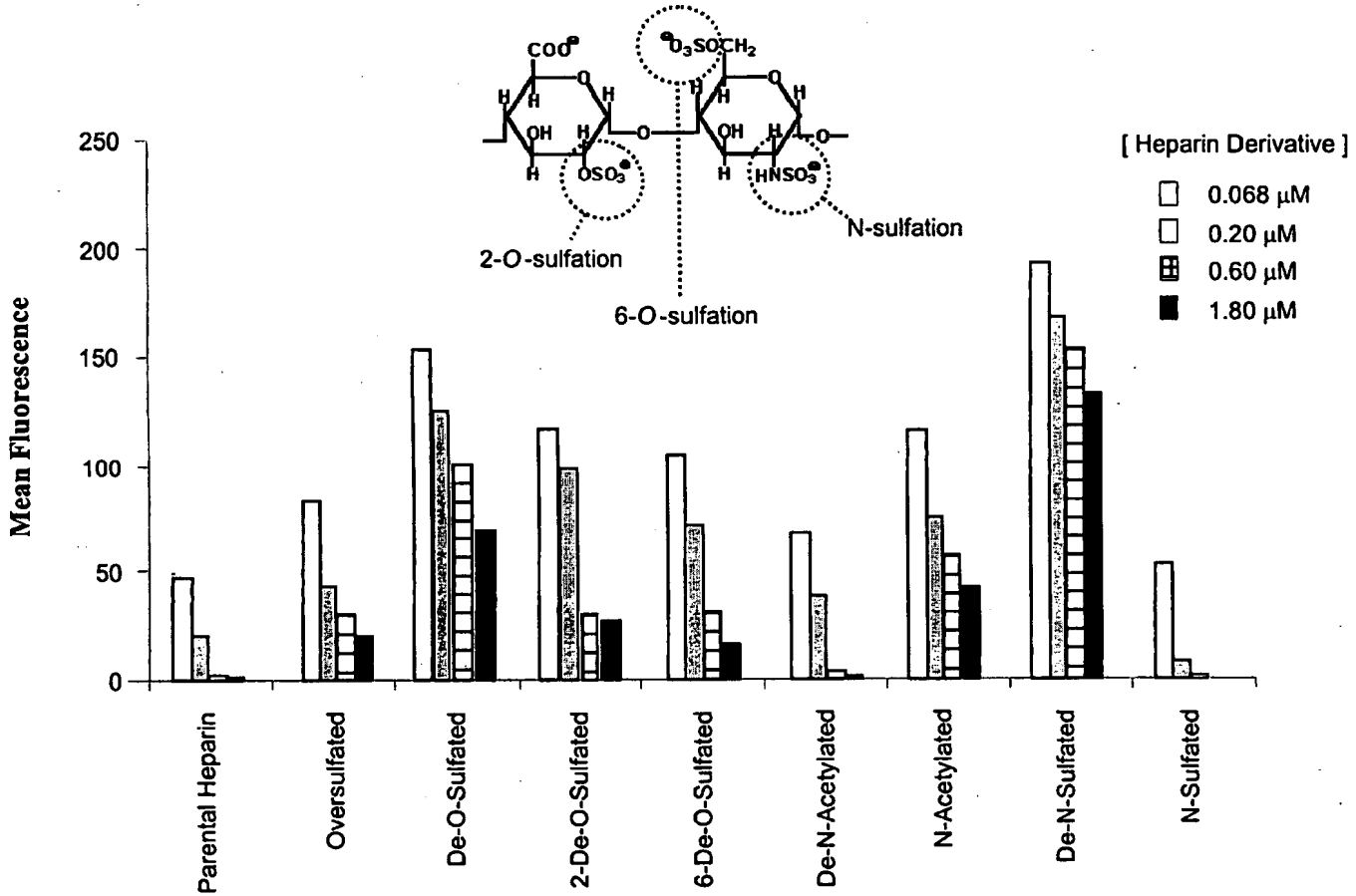

Mean Fluorescence
250
200
150
100
50
0
[Heparin Derivative]
0.068 μM
0.20 μM
0.60 μM
1.80 μM
2-O-sulfation
6-O-sulfation
N-sulfation
Parental Heparin
Oversulfated
De-O-Sulfated
2-De-O-Sulfated
6-De-O-Sulfated
De-N-Acetylated
N-Acetylated
De-N-Sulfated
N-Sulfated

Figure 4

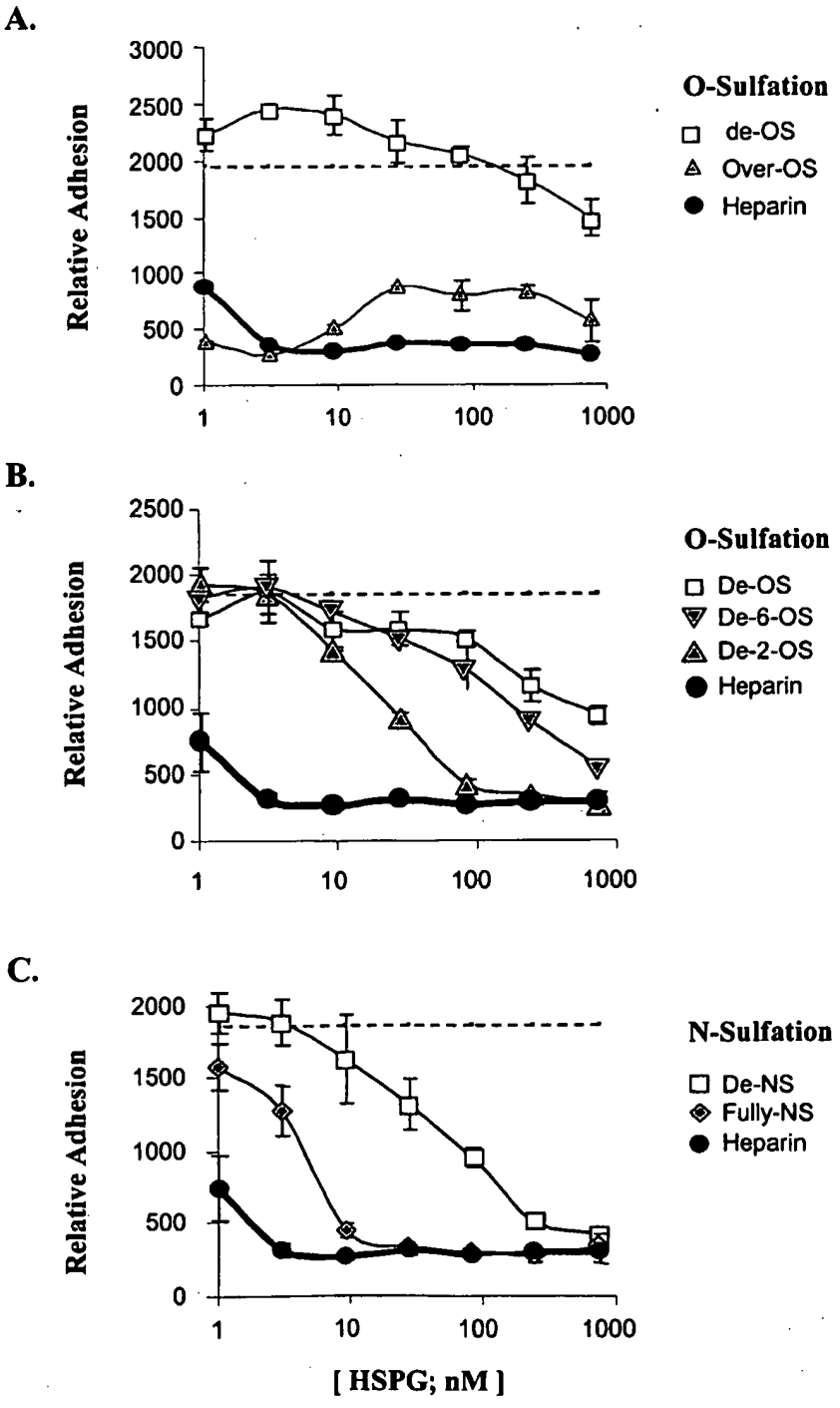

A.
Relative Adhesion
3000
2500
2000
1500
1000
500
0
1
10
100
1000
O-Sulfation
de-OS
Over-OS
Heparin
B.
Relative Adhesion
2500
2000
1500
1000
500
0
1
10
100
1000
O-Sulfation
De-OS
De-6-OS
De-2-OS
Heparin
C.
Relative Adhesion
2000
1500
1000
500
0
1
10
100
1000
[HSPG; nM]
N-Sulfation
De-NS
Fully-NS
Heparin

Figure 5

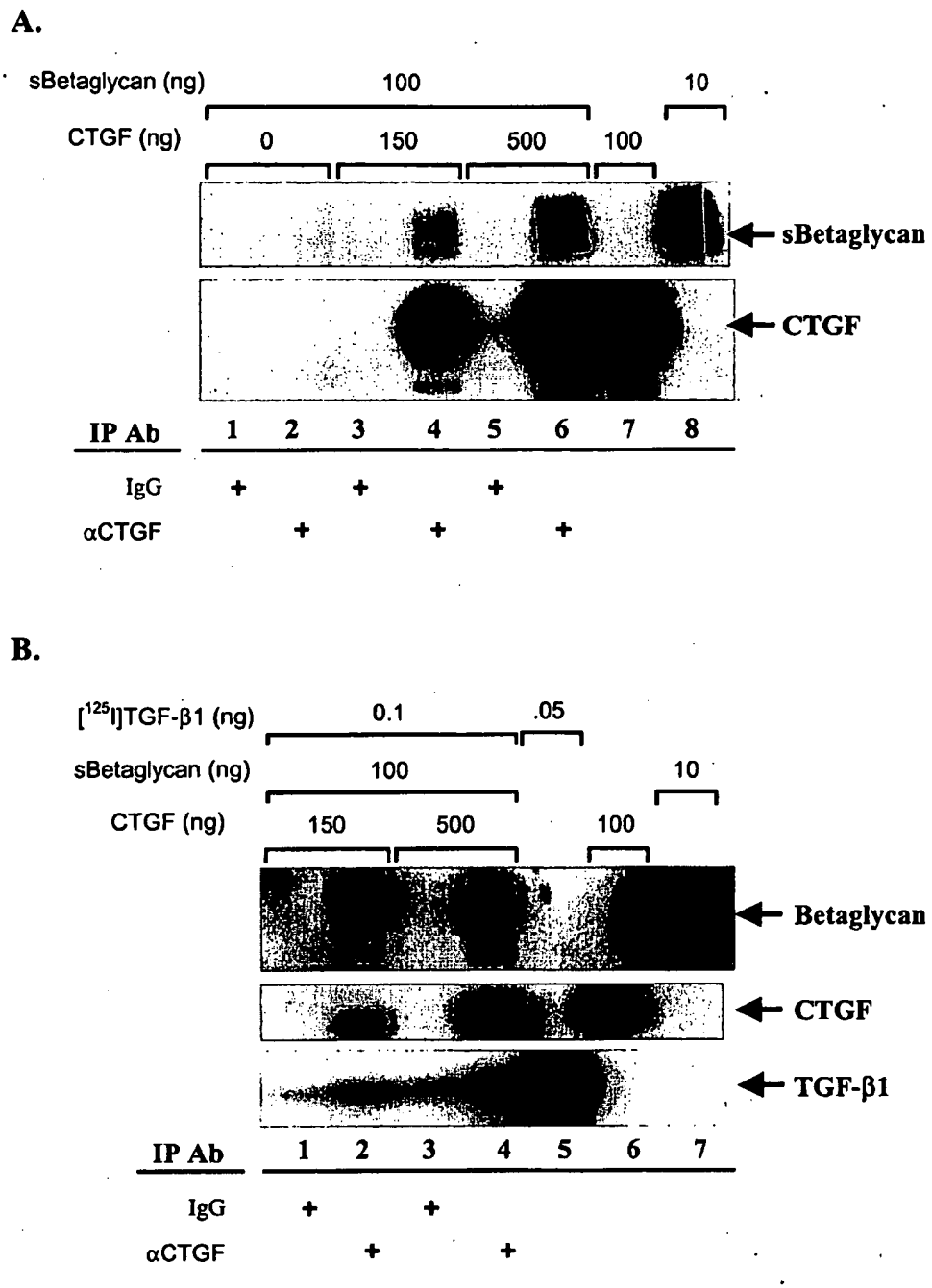

A.
sBetaglycan (ng)
100
10
CTGF (ng)
0
150
500
100
sBetaglycan
CTGF
IP Ab
1 2 3 4 5 6 7 8
IgG
αCTGF
B.
[125I]TGF-β1 (ng)
0.1
.05
sBetaglycan (ng)
100
10
CTGF (ng)
150
500
100
Betaglycan
CTGF
TGF-β1
IP Ab
1 2 3 4 5 6 7
IgG
αCTGF

Figure 6

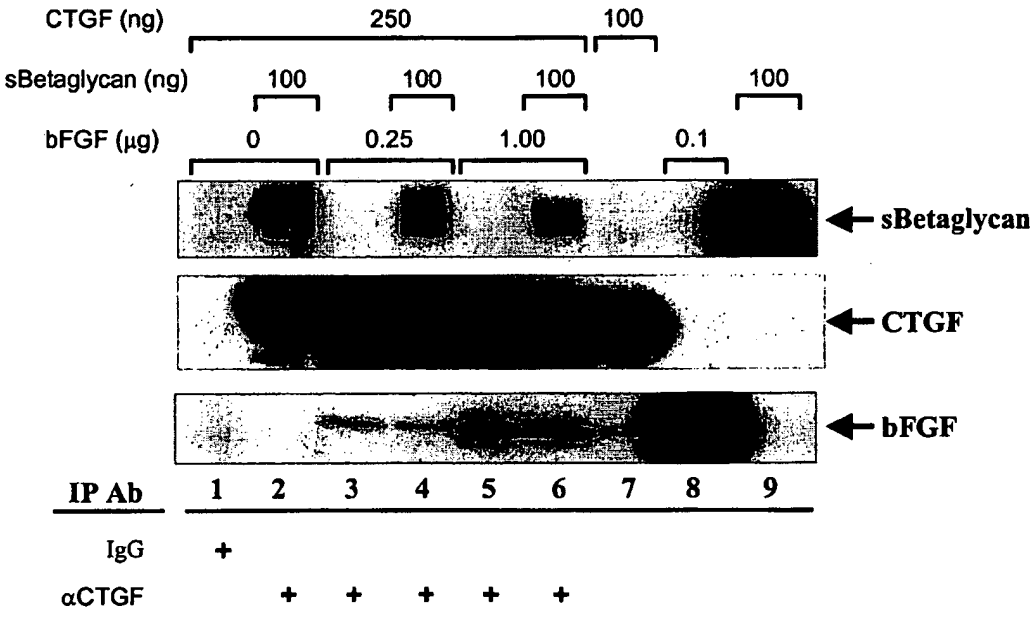

CTGF (ng)
250
100
sBetaglycan (ng)
100
100
100
100
bFGF (μg)
0
0.25
1.00
0.1
sBetaglycan
CTGF
bFGF
IP Ab
1 2 3 4 5 6 7 8 9
IgG
αCTGF

Figure 7

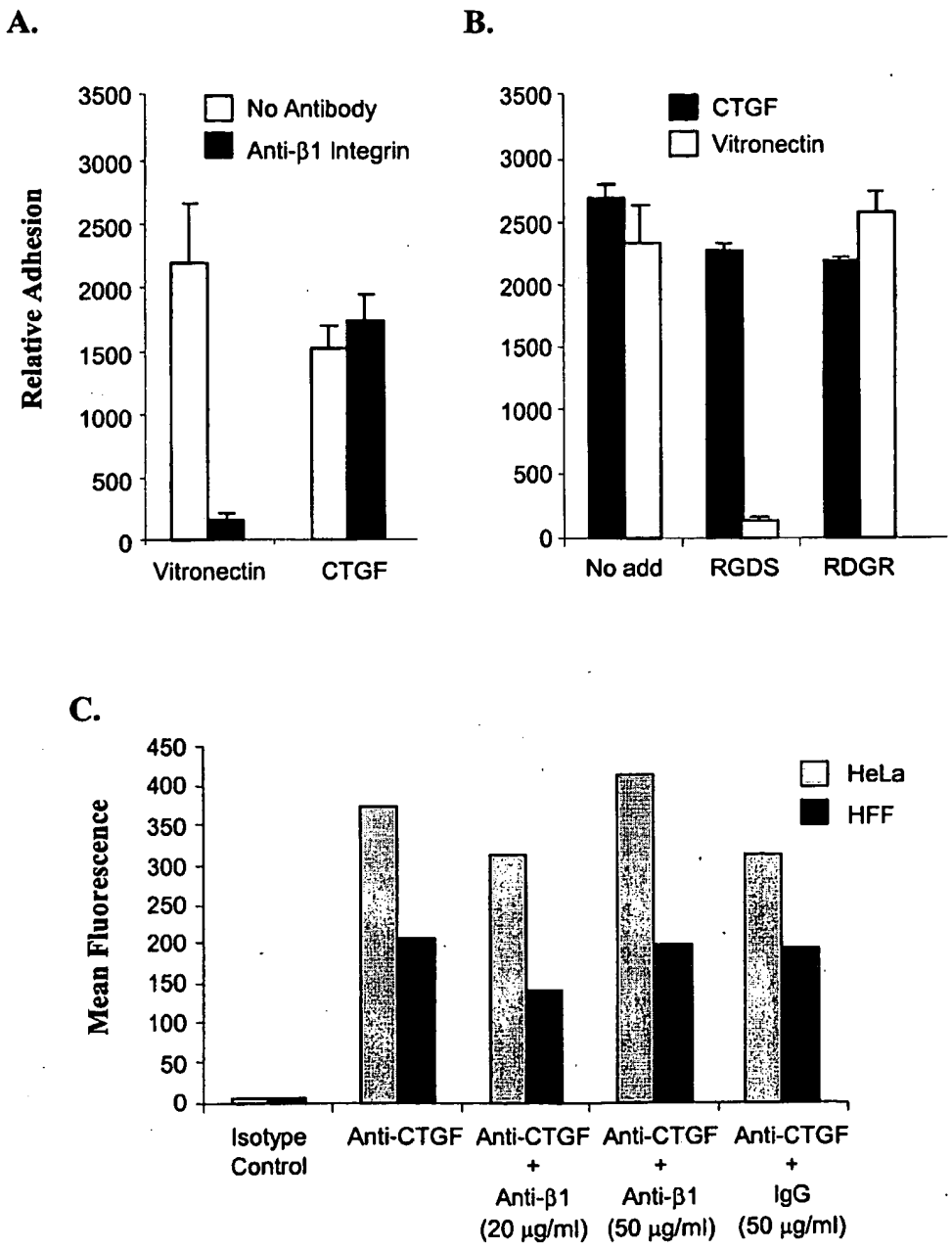

A.
B.
C.
Relative Adhesion
3500
3000
2500
2000
1500
1000
500
0
No Antibody
Anti-β1 Integrin
Vitronectin
CTGF
CTGF
Vitronectin
No add
RGDS
RDGR
Mean Fluorescence
450
400
350
300
250
200
150
100
50
0
HeLa
HFF
Isotype Control
Anti-CTGF
Anti-CTGF + Anti-β1 (20 μg/ml)
Anti-CTGF + Anti-β1 (50 μg/ml)
Anti-CTGF + IgG (50 μg/ml)

Figure 8